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SOLID PHASE PEPTIDE SYNTHESIS

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fully introduced into peptides of many different lengths by means of this reagent. There have been no reports of racemization of *t*-butyloxycarbonyl or *o*-nitrophenylsulfenyl amino acids in diimide-mediated solid phase coupling reactions. The reagent is commercially available, inexpensive, and convenient to use, and promotes very rapid coupling. These advantages have been responsible for its wide application to solid phase, in spite of certain cautions and limitations associated with its use.

The ω-amide functions of asparagine and glutamine may be dehydrated to nitriles by diimides during the coupling reaction (16), and a certain proportion of these ω-cyano derivatives may thus be permanently incorporated into the peptide chain. This side reaction has precluded the use of diimides for the incorporation of glutamine and asparagine residues in solid phase synthesis, since there is no way that these by-products may be conveniently removed. This problem has been successfully overcome by using *p*-nitrophenyl esters to introduce these amino acids into peptide chains. Suitable conditions for these reactions are discussed below.

An undersirable side-reaction in the coupling of all amino acids by means of diimide reagents is rearrangement of the active intermediate to the unreactive acyl urea (Figure 15), which reduces the amount of activated amino acid available for reactions and necessitates the use of an excess of protected amino acid and diimide at each step. The fraction of the reactants lost at each step by this side-reaction depends on the structure of the amino acid being activated, the structure of the amine component of the reaction (the last-introduced amino acid), the solvent, and the concentration of the reactants during the reaction. Bulky amino acid residues, in either the activated component or the amine component, slow down the reaction rate and allow the activated complex more time to rearrange to the undesired acyl urea. The rate of rearrangement is greatly affected by the nature of the solvent, being faster in solvents of high polarity. The control of these factors is well-exemplified by Merrifield's experience in his synthesis of bradykinin (72). He found that when dimethylformamide was the reaction medium, all the amino acids of bradykinin could be completely coupled by the use of two to four equivalents of each amino acid (and of dicyclohexylcarbodiimide) except proline, of which eight equivalents were required. In contrast, when dichloromethane (methylene chloride) was the solvent, 1.5 equivalents of each amino acid was adequate, which is why dichloromethane is used for diimide-mediated coupling reactions whenever possible. As a general routine, 2.5 equivalents of each amino acid and diimide are used in order to provide a greater margin of safety. Three amino acids, the *t*-butyloxycarbonyl derivatives of nitroarginine, tryptophan, and *im*-benzylhistidine, do not dissolve well enough in dichloromethane to allow exclusive use of it. Instead, these protected amino acids are dissolved in the minimum amount necessary of purified dimethylformamide, and the solution is then made up to the desired volume with dichloromethane.

The volume of solvent used in coupling reactions is usually close to the minimum amount needed to suspend the resin as a slurry in the reaction vessel and to allow for proper mixing. This high concentration promotes the desired coupling reaction at the expense of the rearrangement to the acyl urea, which is intramolecular and proceeds at a fixed rate (for any given amino acid, solvent, and temperature) regardless of concentration, whereas the coupling reaction is bimolecular and proceeds faster when the concentration is high. Since the concentration of reactive groups on the resin is fixed, concentration can be effectively increased only by lowering the volume of the solvent which suspends the resin and dissolves the soluble reactant. At the present time, the effect of temperature on the ratio between the rearrangement and coupling rates has not been adequately investigated.

Users of dicyclohexylcarbodiimide should be reminded that it is a potent contact allergen, and that some people have experienced severe reactions to repeated skin contact. Scrupulous

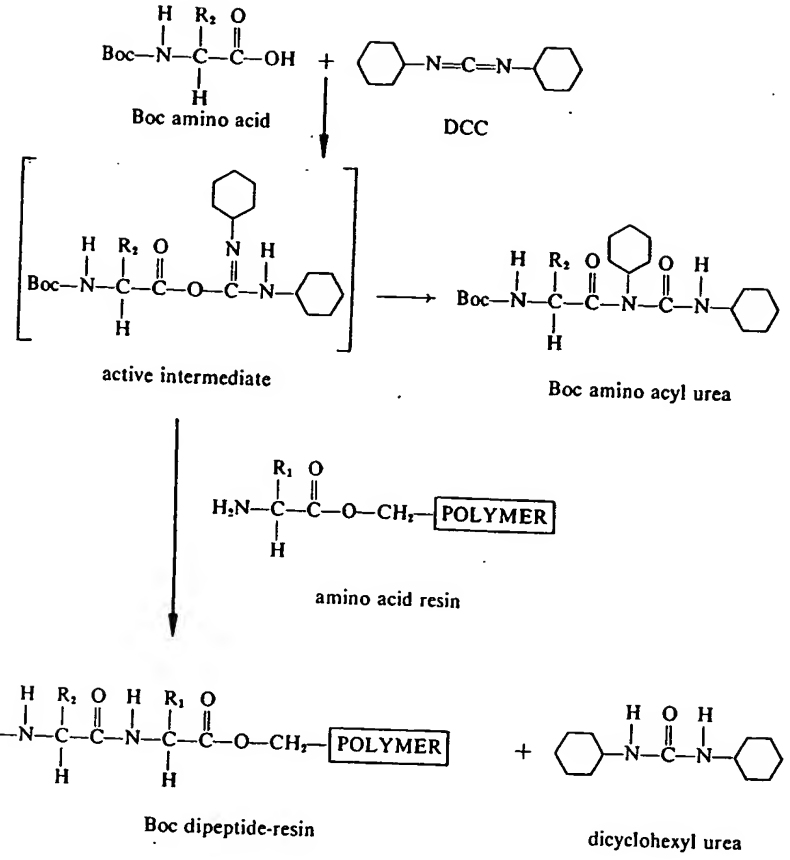

FIGURE 15.
Acyl Urea Formation in Diimide Reactions.

cleanliness must be observed in all areas where the reagent is used, especially in areas around balances. Small bits of diimide carelessly left on balance tables may cause great discomfort to sensitized persons who subsequently use the area. Sensitive persons should routinely wear disposable plastic gloves when handling the reagent.

Active esters of a variety of amino acids have now been used successfully in solid phase synthesis [(14); Figure 16]. As we mentioned above, *p*-nitrophenyl esters of *t*-butyloxycarbonyl asparagine and glutamine are routinely used to introduce these residues into peptides in order to avoid the diimide-induced dehydration of the amide to the nitrile. Nitrophenyl esters have also been used exclusively for the synthesis of several peptides (15), including the entire A-chain of insulin (39). The coupling reactions of these esters are generally slower than diimide-mediated couplings. In the Merrifield laboratory, satisfactory coupling of several amino acid nitrophenyl esters has been obtained with four equivalents of the active ester and a reaction time of four hours, in contrast to the routine use of 2.5 equivalents of reactants and a coupling time of two hours for diimide-mediated reactions. Other investigators have sometimes found it necessary to use even more time. One possible advantage of active esters is that the excess reagent, necessary to promote complete coupling within a reasonable time, should in principle be recoverable from the mixture at the end of the reaction. Where the cost of the reagents is a limitation, this might possibly be a deciding factor, since with the diimide reactions,

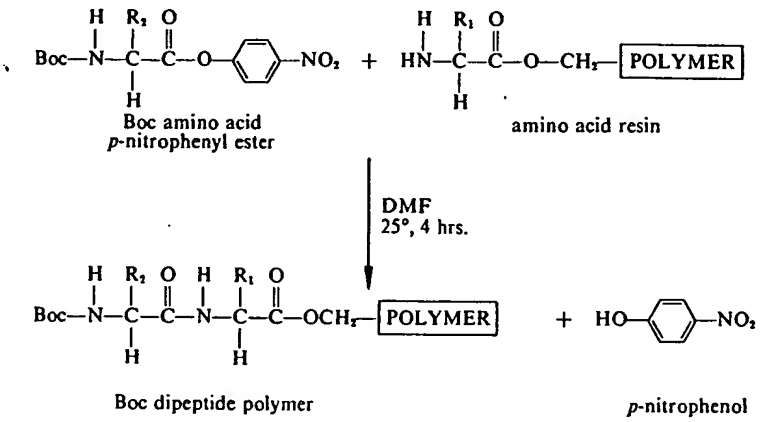

FIGURE 16.
Solid Phase Synthesis with Active Esters.

the excess amino acid is presumably lost as the acyl urea. Beyerman *et al.* (10, 11) have used nitrophenyl esters in the presence of a 1,2,4-triazole catalyst for the solid phase synthesis of oxytocin. Nitrophenyl esters couple satisfactorily only in a dimethylformamide solvent. The dimethylformamide used for this purpose must be carefully purified, since any dimethyl amine present could react with the active ester.

Other active esters have so far been less promising. In a bradykinin synthesis, N-hydroxysuccinimide esters were found to give less satisfactory results than diimide couplings (128). The N-hydroxysuccinimide ester of a dipeptide has been used to lengthen a peptide chain by two amino acids at one step (140). There is one brief report of the use of dicyclohexylcarbodiimide in the presence of N-hydroxysuccinimide (140). Other active esters, the trichlorophenyl and pentachlorophenyl esters of nitrophenylsulfenyl amino acids, were reported to give unsatisfactory results in solid phase (11).

Other coupling agents have been used even less frequently. Woodward's reagent (N-ethyl-5-phenylisoxazolium-3′-sulfonate) has been used for the solid phase synthesis of an angiotensin analog (17). Mixed anhydrides have been used to synthesize a tripeptide (117) and a depsipeptide (118), and in a synthesis involving ε-aminocaproic acid (102). At least one amino acid has been coupled to a peptide-resin by the azide method (142). Although Woodward's reagent may prove useful for solid phase work, the other two methods appear impractical for routine syntheses because of the additional work necessary to prepare the active intermediates.

2

LABORATORY TECHNIQUES IN SOLID PHASE PEPTIDE SYNTHESIS

STARTING MATERIALS

Preparation of the resin

Preparation of chloromethyl resin (73). Swell 50 g of resin beads (polystyrene that has been copolymerized with 2% divinylbenzene; see Appendix C, item 1) by stirring at 25° for 1 hour in 200 ml of $CHCl_3$ in a three-neck ⅘ round-bottom flask, then cool it to 0°. Add a cold solution of 3.8 ml anhydrous $SnCl_4$ in 100 ml of chloromethyl methyl ether from a dropping funnel, while stirring. Continue stirring for 30 min. at 0°. Filter the mixture on a fritted glass Buchner funnel, and wash with 1 liter of 3 parts dioxane to 1 part water, then with 1 liter of 3 parts dioxane to 1 part 3 *N* HCl, allowing several brief periods for the wash solvent to soak into the beads. Wash the beads thoroughly with dioxane, with water, and with methanol, allowing time for each solvent to penetrate the beads. Dry the resin overnight over $CaCl_2$ under high vacuum.

To determine the degree of chloromethylation, heat an aliquot of the resin (200 mg) in 3 ml pyridine in a test tube for 2 hours at 100°. Transfer the mixture quantitatively to a 125-ml Erlenmeyer flask with 30 ml of 50% HOAc, and add 5 ml of concentrated HNO_3. Analyze the chloride by the modified Volhard method (see p. 55), omitting the addition of nitric acid and adding only 5 ml of $AgNO_3$ (0.1 *N*). These conditions for chloromethylation have yielded 0.9 to 2.0 millimoles of Cl per g of resin. The degree of chloromethylation can be controlled by changing the amount of $SnCl_4$ used or the time and temperature of the reaction.

Preparation of nitrochloromethyl resin (71). Slowly add 50 g of chloromethyl resin (1 to 2 millimoles of Cl per g of resin), while stirring, to a beaker containing 500 ml of fuming nitric acid (90% HNO_3, sp. gr. 1.5) prechilled in an ice-salt bath to 0°. Continue stirring at 0° for 1 hour. Pour the mixture onto crushed ice. After the ice melts, filter the resin on a coarse fritted glass Buchner funnel, and wash successively with water, dioxane, and methanol. Dry the resin under vacuum. These conditions have yielded 6.4 millimoles NO_2 group per g of resin, as estimated by Dumas nitrogen analysis. This degree of substitution represents approximately one NO_2 group per aromatic ring.

Preparation of hydroxymethyl resin (83). Cover chloromethyl resin (1 to 2 millimoles of Cl per g of resin; 1 equiv. Cl) and potassium acetate (1.1 equiv.) with methyl cellosolve (ap-

proximately 6 ml per g of resin) and heat in an oil bath at 125° to 135° for 24 hours. The reaction is carried out in a ⅄ 24/40 round-bottom flask fitted with a 500-mm water condenser carrying a $CaCl_2$ drying tube. Filter the acetoxy resin which results from this procedure and wash thoroughly with water and with methanol. The yield of acetoxy resin is measured by titration of chloride in the combined filtrate and wash (see p. 55). This procedure usually gives essentially complete conversion to the acetoxy form. Saponify the acetylated resin by stirring for 48 hours at room temperature with 25 ml per g of resin of a mixture of 2 vols dioxane and one vol 0.5 *N* NaOH. At least 2 equiv. of NaOH should be used for each equiv. of acetoxy groups. Filter the resin, wash with water and then methanol, and dry. The degree of saponification to the hydroxymethyl resin is estimated by back-titration of the aqueous wash with standard HCl. This series of reactions can also be monitored qualitatively by infrared spectroscopy (26, 54). KBr pellets may be made of the polymer beads directly.

Preparation of Boc amino acids

Synthesis of Boc amino acids in a pH-stat: The Schnabel method (110). Suspend 0.05 mole of the amino acid and 0.055 mole of Boc azide in 10 ml of water and 10 ml of dioxane, and place in the vessel of a *p*H-stat (autotitrator). Fill the reservoir of the *p*H-stat with 4 *N* NaOH. Advance the *p*H control of the instrument to the point where continued uptake of base indicates that the reaction is proceeding at a reasonable rate. A few amino acids will react at *p*H 8.5, and most at *p*H 9.8; a few require *p*H 10.2 for a reasonable rate of reaction. The reaction is usually complete in a few hours, although the reaction with certain amino acids is quite sluggish and may require more than 24 hours. Amino acid derivatives containing alkali-labile groups (e.g., esters and amides of aspartic and glutamic acids) should be treated with caution; the reaction should be carried out at the lowest *p*H practical. With these derivatives, purer products will probably be obtained by use of the dimethyl sulfoxide procedure (see p. 29).

The end of the reaction is indicated by cessation of base uptake. Extract the solution 3 times with ether to remove unreacted azide. Chill the aqueous phase in ice, acidify with solid citric acid to *p*H 3, and extract 3 times with ethyl acetate. Certain Boc amino acids (serine, threonine) are quite soluble in water and can not be satisfactorily extracted by the ethyl acetate; if using them, saturate the aqueous phase with NaCl before the extraction. A recent report (70) indicates that better yields may be obtained if the chilled reaction mixture is acidified to *p*H 2 with HCl in a *p*H-stat, then extracted in the usual way with ethyl acetate.

Wash the ethyl acetate extract 3 times with small portions of water (saturated NaCl solution for water-soluble derivatives), dry over $MgSO_4$, and evaporate under reduced pressure. Most Boc amino acids can be crystallized from EtOAc-hexane. Of the commonly used derivatives, Boc methionine and α-Boc-ε-Z-lysine usually fail to crystallize, and are frequently crystallized as dicylcohexylamine salts, but using the latter introduces an extra recovery step before the Boc amino acids can be used in synthesis, and is not recommended.

Boc-*im*-Bzl-His is a zwitterion and cannot be extracted from aqueous acid. For it, adjust the reaction mixture to *p*H 6.0 with solid citric acid and concentrate under reduced pressure until crystallization begins. Chill the suspension for several hours and filter to remove the product.

Purity of the Boc amino acids is most conveniently assessed by TLC (see pp. 58–60). The Boc group is removed from the derivatives by exposure to HCl vapor after development of the plates in the appropriate solvent; the ninhydrin spray can then be used. Boc amino acids, if pure, are stable at room temperature for long periods of time, but any traces of acid remaining in them will promote cleavage of the Boc group and accumulation of free amino acid. A rough measure of whether there is free amino acid is the solubility of the Boc derivative in CH_2Cl_2; most Boc derivatives (except those of

nitroarginine, tosylarginine, *im*-Bzl histidine, and tryptophan) are soluble to the extent of 1 g in 10 ml. An undissolved residue usually indicates presence of free amino acid, which should be removed by filtration. It is especially important that free amino acids not be present in the Boc derivatives, since they will cause undesirable side reactions in the synthesis.

The greatest difficulty is encountered with Boc glutamic acid γ-benzyl ester, since the Boc glutamic acid is almost impossible to separate by precipitation or crystallization. The dimethyl sulfoxide method of synthesis (see below) is preferred for this derivative. The following CCD was developed (78) to purify this derivative:

For the solvent, use: *n*-butanol, 600 ml; pyridine, 100 ml; acetic acid, 10 ml; water, 850 ml.

Set up a CCD system of 7 separatory funnels. For 21 g of crude material, use 175 ml of each phase. Shake and separate the solvent mixture, and place 175 ml of lower phase in each separatory funnel. Place an equal volume of upper phase in funnel 1. Make the run in the usual way, dissolving the sample in funnel 1, and transferring the upper phase to funnel 2, adding fresh upper phase to funnel 1, and so on. The materials which have high partition coefficients are then found at the end of the run in the higher numbered funnels. Here the desired product will be in tubes 5 and 6, with some left in the upper phase of 4. Separate the upper phase of 4, reextract with fresh lower phase, and combine this upper phase with both upper and lower of 5 and 6. Evaporate under reduced pressure to about ¼ volume and shake with 200 ml of EtOAc. Save this EtOAc for later use. Chill the aqueous phase, acidify with 10% citric acid to *p*H 3.5 (50 ml), and extract 3 times with 100 ml of EtOAc. Combine these EtOAc extracts with the previous EtOAc phase, wash 4 times with 200 ml of 2% citric acid, wash 3 times with H_2O, dry over $MgSO_4$, and evaporate under reduced pressure, to obtain 19.2 g of the desired product.

Commercially available *t*-butyloxycarbonyl azide (*t*-butyl azidoformate) has been widely used for the synthesis of Boc amino acids with complete success. If the reader wishes to prepare his own Boc azide, he can use the directions of Carpino (18) for synthesis of *t*-butyl carbazate, and the directions of Carpino *et al.* (19) for diazotization of the carbazate to the Boc azide.

CAUTION: Prepare and handle the Boc azide in a good hood; it is quite toxic. Inhalation of the vapor has caused severe headache and other symptoms in sensitive individuals. *Do not distill the azide;* distillation of it is unnecessary, *and has caused explosions.*

Synthesis of Boc amino acids using magnesium oxide: the Schwyzer method (116). Stir a mixture of the amino acid (20 millimoles), Boc azide (4.3 g, 30 millimoles), magnesium oxide (1.6 g, 40 millimoles), dioxane (60 ml), and water (30 ml) at 40 to 45° for 20 hours. Cool the reaction mixture, remove the magnesium oxide by filtration, and wash it twice with 100 ml of water. Extract the combined filtrate and washings 3 times with ether to remove unreacted azide. Chill the aqueous phase with ice, acidify with solid citric acid, and work up as described in the preceding section.

Synthesis of Boc amino acids in dimethyl sulfoxide (128). Although satisfactory yields of Boc derivatives of most amino acids can be obtained by one of the foregoing procedures, there are a few amino acids for which these methods fail. Since the γ-*p*-nitrobenzyl ester of glutamic acid is completely insoluble in water-containing systems, no product was obtained with the Schwyzer method. On the other hand, the dimethyl sulfoxide procedure gave a satisfactory yield. When glutamic acid γ-benzyl ester is converted to the Boc derivative in aqueous alkaline media, partial hydrolysis of the ester occurs, and the product is contaminated with Boc glutamic acid, which is very difficult to separate from the desired product. Use of such a contaminated product in peptide synthesis can lead to chain crosslinking and incorporation of γ-glutamyl residues in the

peptide. The dimethyl sulfoxide procedure completely eliminated this difficulty.

Stir a mixture of 2.06 g (0.01 mole) of γ-*p*-nitrobenzyl glutamate, 50 ml of dimethyl sulfoxide, 2.7 ml (2.0 g, 0.02 mole) of triethyl amine (sp. gr. 0.732), and 1.5 ml (1.5 g, 0.01 mole) of Boc azide (sp. gr. 1.01) for 20 hours at room temperature. Dilute the solution with 3 volumes of water and extract 3 times with ether to remove any unreacted azide. Chill the aqueous phase, acidify with citric acid, and work up as described in the preceding sections. The product should weigh 3.8 g, and be homogeneous by TLC.

Synthesis of Boc amino acid p-nitrophenyl esters (16). Chill a solution of 4.64 g (0.02 mole) of Boc asparagine and 11.1 g (0.08 mole) *p*-nitrophenol in 15 ml of purified DMF (see p. 31) to 0° in an ice-salt bath, and add a solution of 4.6 g (0.022 mole) of DCC in 4 ml of DMF in portions. Let the mixture stand overnight at 4°. Remove the crystallized dicyclohexyl urea by filtration and wash with cold DMF. Evaporate the combined filtrate and washings under high vacuum from a 35° bath. Dilute the oily residue, which usually begins to crystallize, with 200 ml of absolute ether. Filter the colorless crystals and wash well with ether. The product will be pure; m. p. 163°. The large excess of *p*-nitrophenol promotes a high yield of the desired product, and the excess is easily removed from the product. If the Boc amino acid to be esterified is freely soluble in EtOAc, use the latter for the synthesis.

Preparation of special reagents

Preparation of 4 N HCl in dioxane. The equipment for preparing HCl-dioxane or HCl-HOAc is shown in Figure 17. Place dioxane (200 ml), purified through Al_2O_3 (see p. 31), in a 250-ml, graduated, pressure-equalizing separatory funnel. Bubble dry HCl from a cylinder through an empty safety trap into the separatory funnel by a gas inlet adapter, and attach a $CaCl_2$ drying tube to the outlet. *Watch the gas flow carefully; do* NOT *leave the operation unattended.* If HCl is passed into the dioxane nearly as fast as it can be absorbed (so that the bubbles disappear before reaching the top of the liquid), the temperature of the solution will usually rise to about 50°. When the dioxane seems to be about saturated at this temperature, close the HCl tank, clamp off the HCl inlet, and cool the HCl-dioxane to room temperature. To determine the normality of the reagents, which should be 4.0 to 4.1, dilute an aliquot of the HCl-dioxane 1:10 with water, and titrate 1 ml of this dilute acid with 0.1 *N* NaOH, using phenolphthalein as the indicator. If the HCl is too concentrated, add the calculated amount of dioxane to the separatory funnel to give the proper concentration. After mixing, retitrate to check that the proper dilution was made. To prevent loss of HCl on standing, the top of the reservoir must be tightly closed at all times, by removing the gas inlet tube and stoppering with a polyethylene ⅋ stopper. The needle valve should be removed from the gas cylinder at the end of the operation, washed well with methanol, and dried thoroughly.

CAUTION: The entire operation must be done in a well-ventilated hood.

Preparation of 1 N HCl in glacial acetic acid. The procedure and setup for preparing 1 *N* HCl in acetic acid are the same as for preparing 4 *N* HCl-dioxane. Be sure to observe the precautions given there. Place reagent-grade HOAc (200 ml) from a freshly opened bottle in a pressure-equalizing separatory funnel, and bubble HCl into the acetic acid until the solution appears saturated at room temperature. Determine the concentration of HCl by the Volhard chloride-assay method (see p. 55). The HCl concentration at this stage is usually 1 to 1.1 *N*; higher values indicate that the HOAc is not anhydrous. Keep the separatory funnel tightly stoppered.

CAUTION: The entire operation should be done in a well-ventilated hood.

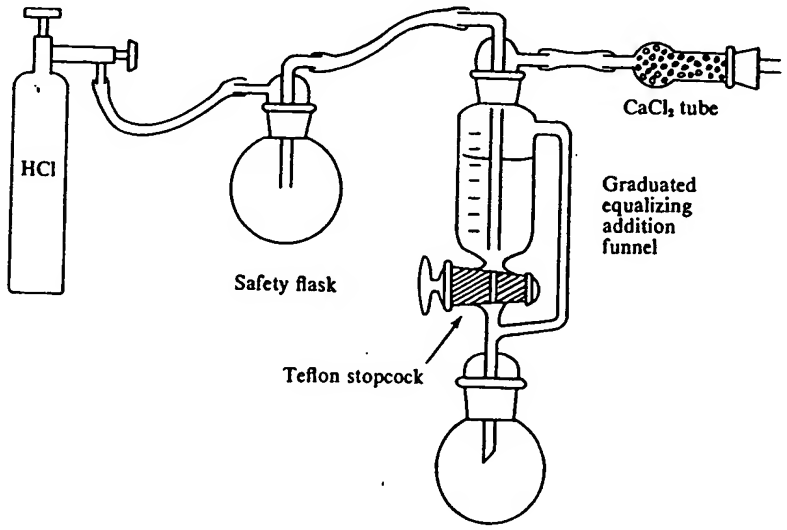

FIGURE 17.
Apparatus for HCl-Dioxane Preparation.

Purification of solvents

Preparation of peroxide-free dioxane. Pour dioxane through a 10 by 40 cm column of aluminum oxide (alumina, Al_2O_3). Use the column until the effluent begins to give a positive peroxide test, performed as follows: mix dioxane with an equal volume of 4% aqueous KI; the dioxane is considered peroxide-free if the solution is colorless after one minute. Purified dioxane may be stored under nitrogen in brown bottles in the cold. An alternate procedure for storing the column-purified solvent is to keep a little alumina in the bottom of the storage bottle and then filter the dioxane just before use.

CAUTION: Dioxane is reported to be toxic. Do not breathe vapors excessively.

Purification and analysis of dimethylformamide (128, 136). DMF is *very* difficult to obtain dry and free of dimethylamine. All commercial DMF, even spectroquality reagent, has been found to contain large amounts of dimethylamine.

Shake 2 liters of commercial DMF with dry KOH pellets, then decant into a round-bottom, 5,000 ml ⅀ flask with an equal volume of dry benzene (keep benzene over KOH pellets). If the KOH becomes very wet from water in the DMF, repeat the KOH drying step. Place the flask in a heating mantle, and attach a fractionating column and distilling head with appropriate condenser and adapters for collecting solvent by using an H_2O aspirator vacuum (items 8, 13, 14, Appendix B); place a drying tube and a vacuum gauge between the aspirator and the distillation apparatus. Distill the benzene without vacuum, then the DMF with vacuum. (DMF decomposes slightly when distilled at 1 atm.) Take fractions and test with dinitrofluorobenzene (FDNB) for the presence of dimethylamine as follows: mix an equal volume of FDNB solution (1 mg per ml in 95% alcohol) and DMF; let stand 30 min.; read the absorbance at 381 mμ. Blank FDNB, 0.5 mg per ml, usually reads approximately 0.2; good DMF should have a net A_{381} no higher than 0.1 to 0.15. DMF having a slightly higher absorbance can be used in the wash before and after the coupling reaction. Place the good

DMF fractions in brown bottles which should be flushed with N_2 and stored in the cold. Bottles may be sealed with rubber stopples by using a sealing device; the DMF may then be removed with a hypodermic syringe. Allow bottles to warm to room temperature before opening to avoid condensation of atmospheric water.

SOLID PHASE SYNTHESIS OF PEPTIDES

Attachment of the first amino acid to the resin

Attachment to the chloromethyl resin (72). Combine the Boc amino acid desired as the carboxyl residue in the peptide to be synthesized (1.0 mole), the chloromethyl resin (1.0 mole of Cl), and triethyl amine (0.9 mole; 0.14 ml per millimole; sp. gr. 0.723), and reflux gently in absolute EtOH for 24 to 65 hours in an oil bath heated to approximately 90°. EtOAc can be used as the refluxing solvent, and *must* be used when Boc amino acids are esterified to the nitro resin. Use enough alcohol to cover the resin (usually 2 to 5 ml per g of chloromethyl resin). Add the triethyl amine to the mixture last. Perform the reaction in a round-bottom ⅋ 24/40 flask fitted with a 500-mm H_2O condenser with a $CaCl_2$ drying tube attached. Although Merrifield stirs the reaction mixture with a magnetic stirrer, it is not essential. Most Boc amino acids are refluxed 24 hours, but Boc nitroarginine is refluxed 65 hours. The following Boc amino acids have so far been attached to the resin by this procedure: Ala, NO_2-Arg, β-Bzl-Asp, Asn, S-Bzl-Cys, γ-Bzl-Glu, Dnp-His, Gln, Gly, Ile, Leu, α-Z-Lys, ϵ-Z-Lys, Phe, Pro, O-Bzl-Ser, Thr, O-Bzl-Thr, Trp, O-Bzl-Tyr, Val. Acylating resin with Bzl-His and Met requires the special techniques described in the next two sections. Boc-Asn was esterified in EtOAc to avoid alcoholysis of the amide. Filter the resin in a tared, coarse-fritted Buchner funnel and wash successively with EtOH, H_2O, MeOH, and CH_2Cl_2, three times with each solvent, allowing adequate contact time for the solvent to penetrate the resin beads and for solutes to diffuse out of the beads. Transfer the resin, still suspended in CH_2Cl_2, to a separatory funnel, stir with CH_2Cl_2 (approximately 20 ml per g), and let it stand until the bulk of the resin floats to the top. Run the solvent, carrying the suspended finest particles of resin, out of the funnel and discard. Repeat this floatation three times more, each time allowing the resin to stand until a fairly sharp demarcation line appears at the bottom of the floating resin, but some particles are still suspended down in the CH_2Cl_2. Return the Boc amino acid resin which has thus been freed of fine particles to the Buchner funnel. Removal of fine resin particles in this way prevents troublesome clogging of the filter disc in the synthesis vessel. After removing all volatile solvents with a water aspirator, dry the resin overnight in a desiccator using a vacuum pump. Weigh the aminoacyl-resin, and hydrolyze about 10 mg to determine the degree of substitution of amino acid on the resin (see pp. 53–54 for details of hydrolysis and amino acid analysis methods). Yields obtained have been 0.1 to 0.5 millimoles per g of resin, using resins having 1.0 to 1.8 milliequivalents of Cl per g, which is a desirable range of substitution. Schwarz Bioresearch reports that resin with much higher substitution also gives satisfactory results. One should not try to replace all of the Cl with amino acid. As we explained on p. 7, since some Boc amino acid may be bound to quaternary amine groups on the resin by ion exchange, the values obtained on hydrolysis may be slightly higher than the actual amount esterified. For greatest accuracy, the Boc amino acid resin should be deprotected with HCl-dioxane before hydrolysis and analysis.

Attachment of Boc-im-Bzl-histidine to the hydroxymethyl resin using dicyclohexylcarbodiimide (83). Suspend hydroxymethyl resin (1.1 g, 0.49 milliequivalents of OH per g) in 10 ml of purified DMF, and add 0.54 g (1.6 millimoles)

of Boc-*im*-Bzl-His. After a few minutes stirring, add 0.33 g (1.6 millimoles) of DCC; stir the mixture overnight at room temperature, with exclusion of moisture. Collect the resin in a fritted glass Buchner funnel and wash thoroughly with DMF, EtOH, and CH_2Cl_2. Dry the resin thoroughly *in vacuo* and analyze for amino acid content by hydrolysis of an aliquot (see pp. 53–54).

CAUTION: Before this aminoacyl-resin can be used for peptide synthesis involving diimide-mediated coupling steps, the remaining free hydroxyl groups on the resin must be covered by acetylation, as described below. The acetylation is not necessary if the entire procedure involves only active esters. This acetylation procedure is also useful for acetylating peptides on the resin.

Transfer the Boc-*im*-Bzl-His-resin, prepared as above, to a solid phase synthesis vessel and rock for 5 min. with 10 ml of purified DMF. Estimate the amount of free hydroxyl groups remaining on the resin from the difference between the hydroxyl content of the hydroxymethyl resin and the amount of histidine coupled to the resin. Add approximately 10 moles of acetic anhydride (mol wt 102, d 1.09) to the resin suspension for every mole of free hydroxyl groups remaining. Rock the suspension 3 min., add an amount of triethyl amine (mol wt 101, d 0.723) equivalent to the acetic anhydride, then rock the resin suspension for 2 hours. Wash the resin 3 times each with DMF, EtOH, and CH_2Cl_2. Remove fine resin particles by floatation as described in the preceding section, then transfer the resin to a fritted glass Buchner funnel, and dry *in vacuo*.

For esterification of Boc methionine to the resin, use the carbonyldiimidazole procedure described in the next section. Boc-Dnp-histidine can be coupled to the chloromethyl resin by the usual procedure, described in the preceding section.

Attachment to the hydroxymethyl resin using N,N'-carbonyldiimidazole (15). Place the Boc amino acid desired as the carboxyl residue in the peptide to be synthesized (3 moles), the hydroxymethyl resin (1.0 mole OH), and N,N'-carbonyldiimidazole (3 moles) in purified DMF (approximately 10 ml per g of resin) in a ⅄ round-bottom flask equipped with a drying tube and stir magnetically overnight. Filter the resin on a fritted glass Buchner funnel and wash thoroughly with DMF, EtOH, and CH_2Cl_2. Remove fine resin particles by floatation, then dry the resin thoroughly *in vacuo* and analyze for amino acid by hydrolysis of an aliquot (see pp. 53–54).

CAUTION: Before this aminoacyl-resin can be used for peptide synthesis involving diimide- or carbonyldiimidazole-mediated steps, the excess remaining free hydroxyl groups on the resin must be covered by acetylation; see the preceding section. If the entire synthesis will be performed by means of nitrophenyl esters, acetylation is not necessary.

Carbonyldiimidazole is quite sensitive to water, so all solvents should be carefully dried before use. This procedure, but using EtOAc as solvent, is the only one found to be satisfactory for esterification of Boc methionine to the resin (60).

Stepwise synthesis of peptides on the resin (79)

Figure 18 shows the apparatus used for the manual synthesis of peptides. (For exact descriptions of the reaction vessels and the mechanical shaker, see pp. 65–68.) The proper size for the reaction vessel is determined by the amount of resin to be used in the synthesis. The large vessel accommodates 5 to 10 g of resin; the medium vessel will handle 1.5 to 4.5 g of resin; the small vessel is used for amounts of 1 g or less. Load the Boc amino acid-resin into the reaction vessel and let it swell in dioxane for a few minutes before beginning the sequence of steps involved in building up the desired peptide. Add the solvents and reagents to the reaction vessel through the ⅄ 14/20 sidearm, and

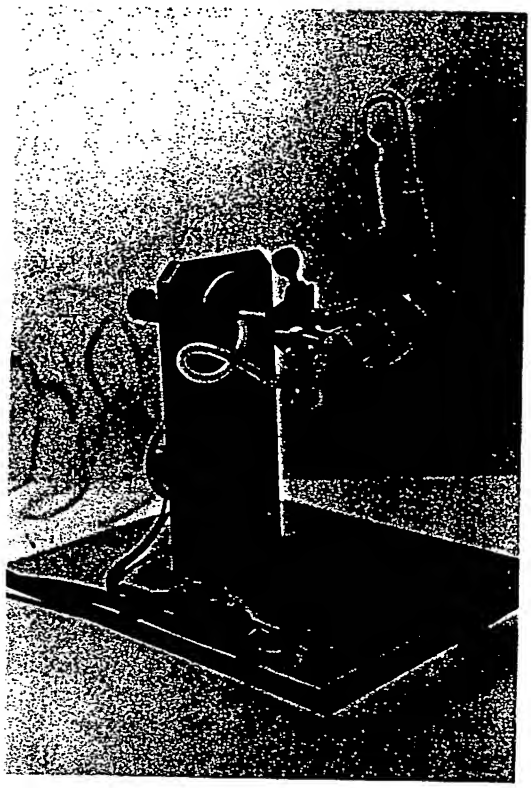

FIGURE 18.
The Shaker and Vessel Used for Solid Phase Peptide Synthesis.

remove by aspiration to a large suction flask through the sintered disc at the bottom of the vessel after placing a drying tube in the sidearm. Charge the drying tube (Appendix B, item 16) with $CaCl_2$, after placing a small amount of indicator Drierite in the bulb of the tube. Keep the ⅋ stopper in the vessel at all other times, except during addition or removal of solvents.

The sequence of steps used to add each amino acid to the resin by the DCC reaction is outlined and explained in schedule A (pp. 38–39). Those who would like to follow a detailed description written in the manner of *Organic Syntheses* should refer to the directions by Merrifield and Corigliano (79) for synthesis of a tetrapeptide.

It is not always easy to carry out a long sequence of many simple, repetitive operations, such as are required in solid phase synthesis, without making a mistake. Unless an accurate, up to the minute record is kept, it is easy to become confused. We have found that the most satisfactory way to keep track of what one is doing is to fill out a data sheet as one performs each operation. (A typical data sheet for the synthesis of a pentapeptide is given on page 36.) As each operation is performed, the chart is checked and the time noted in the appropriate box.

The Boc group can also be removed with 1 *N* HCl-HOAc (see p. 18) instead of 4 *N* HCl-dioxane, but both methods have disadvantages. Whereas HOAc must be completely removed before addition of the diimide, since any trace of HOAc would cause acetylation of the amino acid on the resin instead of coupling of the desired next amino acid residue, dioxane accumulates peroxides, and so must be purified before use (see pp. 18, 31). If HCl-HOAc is used instead of HCl-dioxane, schedule A must be changed as follows: Step 1—HOAc wash, 3 times. Step 2—1 *N* HCl-HOAc. Step 3a—HOAc wash, 3 times. Step 3b (an additional step)—EtOH wash, 3 times. The EtOH wash is added to remove the HOAc. All traces of alcohol must be removed before addition of Et_3N, or peptide may be lost from the resin by transesterification (see p. 12).

Use purified DMF in the diimide coupling reaction instead of CH_2Cl_2 if the amino acid derivative is insoluble or only sparingly soluble in CH_2Cl_2; such derivatives are Boc-NO_2-arginine, Boc-tosyl-arginine, Boc-tryptophan, and Boc-Bzl-histidine. Dissolve the Boc amino acid in the minimum amount of DMF required, then make up the rest of the volume with CH_2Cl_2. DMF should be used only where necessary, since it is difficult to obtain free of dimethylamine, and the rearrangement of the reactive intermediate (see Fig. 15) to the unreactive acyl urea is faster in DMF than in CH_2Cl_2. If the coupling reaction is performed in DMF, the wash solvent before and after the

coupling step (steps 7 and 10 in schedule A) should be DMF instead of CH_2Cl_2.

The DCC-mediated coupling reaction is routine. However, if the active ester is used, such as in coupling Gln or Asn to peptide-resins, certain changes in the schedule are required. The sequence of steps used for coupling active esters is given in schedule B (p. 40). Even though it is hard to obtain DMF of adequate purity, DMF must be used instead of CH_2Cl_2 for *p*-nitrophenyl esters, since these amino acid derivatives do not couple in CH_2Cl_2. The larger excess of Boc amino acid active ester, the increased reaction time, and the small volume of DMF during the coupling reaction all compensate for the slower active-ester reaction.

At the end of the synthesis, remove the resin to a tared, fritted glass funnel, wash with HOAc and EtOH, and dry in a vacuum desiccator over KOH. Since some amino acid seems to be bound to the peptide-resin by ion exchange or adsorption after the coupling step, amino acid analyses of peptide-resins or of crude cleaved peptides will be more accurate if the synthesis is continued through an additional deprotection step. The HCl-dioxane (or HCl-HOAc) removes the adsorbed Boc amino acid. Take great care to wash the peptide-resin free of all excess HCl before it is stored. Record the weight of the peptide-resin. The increase in weight of the resin during the synthesis is a rough measure of the amounts of amino acids incorporated, and indicates the upper limit of the weight of peptide that might be obtained. An aliquot of the resin may be analyzed for its amino acid composition (see p. 53). The peptide is then cleaved from the resin by one of the methods described on pp. 40–46.

Peptides can very easily be synthesized at the rate of two residues a day. With efficient operation, diimide couplings can be easily carried out at the rate of three a day, especially if the third coupling reaction is allowed to continue overnight, which causes no harm. A timer may be used to turn the motor off at the end of the desired reaction time.

A TYPICAL DATA SHEET

Synthesis of: *Leu-Asp-Ala-Gly-Arg-resin*

Boc-nitroarginine-resin (*0.5 g, 284* millimoles per g of resin. Total amino acid, *0.142* millimoles) was placed in the *small* reaction vessel and rocked for 5 min. in *10* ml dioxane. Used *2.5* moles of Boc amino acid and DCC per mole of *Arg*-resin. DCC reagent: *50% in CH_2Cl_2, wt/vol (1 g DCC made up to 2 ml)*.

Calculations:

mg Boc amino acid used

= (mmoles *Arg*-resin) × (molar excess) × (mol wt of Boc AA)
= (*0.142* mmoles) × (*2.5*) × (mol wt of Boc AA in mg/mmole)
= (*0.362*) × (mol wt of Boc amino acid in mg).

ml DCC reagent

= (mmoles *Arg*-resin) × (molar excess) × (mol wt of DCC) × (solution factor)
= (*0.142* mmoles) × (*2.5*) × (206 mg/mmole) × (*2 ml/1000 mg*)
= *0.15 ml.*

AA	Reagent / Vol. / Time / Date	Dioxane wash, 3 times *10* ml each	4 *N* HCl-dioxane *10* ml 30 min.	Dioxane wash, 3 times *10* ml each	$CHCl_3$ wash, 3 times *10* ml each	Et_3N: $CHCl_3$ (1:9) *10* ml (*1* + *9*) 10 min.	$CHCl_3$ wash, 3 times *10* ml each	CH_2Cl_2 wash, 3 times *10* ml each	*Boc-AA in CH_2Cl_2 *6* ml 5 min.	DCC *0.15* ml 2 hours	CH_2Cl_2 wash, 3 times *10* ml each	*mg of Boc amino acid
Gly	*5-24-66*	✓✓✓	*11:05-11:35*	✓✓✓	✓✓✓	*11:45-11:55*	✓✓✓	✓✓✓	*12:05-12:10*	*12:15-2:15*	✓✓✓	*0.362 × 175 = 63.4 mg*
Ala	*5-24-66*	✓✓✓	*2:38-3:08*	✓✓✓	✓✓✓	*3:40-3:50*	✓✓✓	✓✓✓	*3:55-4:00*	*4:00-6:00*	*5-25-66* ✓✓✓	*0.362 × 189 = 68.5 mg*
Bzl-Asp	*5-25-66*	✓✓✓	*10:35-11:05*	✓✓✓	✓✓✓	*11:15-11:25*	✓✓✓	✓✓✓	*11:45-11:50*	*11:50-2:30*	✓✓✓	*Bzl-Asp* *0.362 × 323 = 117 mg*
Leu	*5-25-66*	✓✓✓	*3:05-3:35*	✓✓✓	✓✓✓	*4:40-4:50*	✓✓✓	✓✓✓	*4:55-5:00*	*5:00-7:00*	*5-26-66* ✓✓✓	*0.362 × 231 = 83.6 mg*

Peptide-resin washed in HOAc and EtOH, and dried. Wt = 0.63 g.

Schedule A for solid phase peptide synthesis (diimide coupling)

Step	*Reagent*	*Vol (ml)*	*Time (min.)*
1.	Dioxane wash (3 times)	10	5
2.	4 *N* HCl-dioxane	10	30
3.	Dioxane wash (3 times)	10	5
4.	Chloroform wash (3 times)	10	5
5.	Et_3N-chloroform	10	10
6.	Chloroform wash (3 times)	10	5
7.	CH_2Cl_2 wash (3 times)	10	5
8.	Boc-AA in CH_2Cl_2	7	5
9.	Diimide in CH_2Cl_2	–	120
10.	CH_2Cl_2 wash (3 times)	10	5
		Total	195

INSTRUCTIONS FOR SCHEDULE A

For most rapid operation, in steps 1, 3, 4, 6, 7, and 10 shake the vessel manually between each of the three solvent washes until the resin is evenly suspended. Five minutes is usually sufficient for the three washes in this case. Add the wash solvents so that the entire inner wall of the vessel is rinsed each time, by adding part of the wash solvent so that it flows over the inner wall of the vessel with the vessel tipped slightly to one side of the horizontal, and the remainder with the vessel tipped in the other direction. Rock steps 2, 5, 8, and 9 mechanically on the peptide shaker. *All* the resin must be thoroughly washed down from the vessel walls and equilibrated with each solvent and reagent.

The wash volume is adjusted proportionally to the amount of resin used. The 10 ml wash volume is used for the small reaction vessel, designed for 1 g of resin. The wash volume for the large reaction vessel, designed for 7 to 10 g of resin, is 30 to 60 ml.

STEP 1. *Dioxane wash (3 times).*

STEP 2. *4* N *HCl-dioxane.* (See p. 30 for its preparation.)

Some CO_2 is liberated during this deprotection step. For large batches of resin, vent the vessel and retighten the stopper after most of the CO_2 has been evolved.

If Nps amino acids are used, make the following changes: In step 1, use chloroform as the wash solvent. In step 2, the reagent for deprotection is chloroform plus enough 1 *N* HCl in HOAc so that 3 equiv. of HCl are added to the vessel for each equiv. of Nps groups present. The time of deprotection is 10 min. In step 3, wash the peptide-resin 3 times each with chloroform, DMF, and EtOH (88). If tryptophan is present in the peptide, Nps groups must not be removed by HCl. See p. 49 for details of the thioacetamide procedure (45, 60), which is suitable.

Removal of Boc groups from some amino acids with HCl-dioxane requires full 4 *N* strength reagent for the 30-minute treatment time. Since the reagent is diluted somewhat by the solvent already in the resin, the deprotection conditions may be marginal. Some workers now use a prewash with HCl-dioxane (one-minute shaking), followed by the usual 30-minute treatment. This is especially important with automatic instruments, where there is additional dilution of the reagent by solvent in the connecting tubing.

STEP 3. *Dioxane wash (3 times).*

STEP 4. *Chloroform wash (3 times).*

Peptide-resin may be removed after this step for use in another peptide synthesis or for analysis. Sometimes an EtOH wash is added after the dioxane wash and before resin is removed, since $CHCl_3$ suspensions of resin may be difficult to handle. After a final deprotection (see p. 7), wash the resin at least 6 times with EtOH.

STEP 5. *Et_3N-chloroform.*

The reagent is prepared by adding 1 vol of reagent grade triethyl amine to 9 vol of reagent chloroform. Mix the reagent just before adding it.

STEP. 6. *Chloroform wash (3 times).*

STEP 7. *CH_2Cl_2 wash (3 times).*

STEP 8. *Boc-AA in CH_2Cl_2.*

The number of moles of Boc amino acid is usually 2.5 times the number of moles of the first amino acid on the resin. Larger excesses of Boc amino acid and DCC have been used where steric hindrance has caused lower yields in the coupling reaction. The volume of solvent should be kept low during the coupling step to promote the coupling reaction and minimize the rearrangement to the acyl urea by-product.

STEP 9. *Diimide in CH_2Cl_2.*

The quantity of DCC (1 g made up to 2 ml with CH_2Cl_2) used is equimolar to the Boc amino acid added at step 8. If the DCC contains some dicyclohexyl urea (precipitate in the CH_2Cl_2 solution), the amount may be increased slightly. Prolongation of the coupling reaction beyond 2 hours is of questionable advantage, since the active intermediate may have all rearranged to acyl urea in this time. If there is evidence of poor coupling, it may be more profitable to repeat the coupling reaction with fresh quantities of Boc amino acid and DCC. Before doing so, one may find it advantageous to wash the peptide-resin 3 times with HOAc to remove precipitated acyl urea, then wash it with EtOH, then proceed with step 4.

WARNING: DCC may cause severe allergic reactions in sensitive persons. Avoid all contact with the skin or mucosa. Wash immediately with acetone after any contact. In order to prevent DCC contamination of the balances, transfer the DCC in the hood over disposable paper into a tared vial. Reweigh the vial with DCC in it, and add 1 ml CH_2Cl_2 for every g of DCC; this gives a 50% DCC solution. Prepare enough DCC reagent for the entire synthesis. *Do not pipette the DCC solution by mouth.*

STEP 10. *CH_2Cl_2 wash (3 times).*

When left overnight, the peptide-resin is left at this stage, suspended in CH_2Cl_2.

Schedule B for solid phase peptide synthesis (active ester coupling)

Step	*Reagent*	*Time (min.)*
1.	Dioxane wash (3 times)	5
2.	4 *N* HCl-dioxane	30
3.	Dioxane wash (3 times)	5
4.	Chloroform wash (3 times)	5
5.	Et_3N-chloroform	10
6.	Chloroform wash (3 times)	5
7.	DMF wash (3 times)	5
8.	Boc-AA active ester in DMF	240
9.	DMF wash (3 times)	5
	Total	310

INSTRUCTIONS FOR SCHEDULE B

STEPS 1 TO 6.

Since steps 1 to 6 are the same in schedules A and B, the general instructions and the instructions for these steps are the same for both schedules.

STEP 7. *DMF wash (3 times).*

Use DMF of the highest obtainable purity in the last wash before adding the Boc amino acid active ester and in the coupling reaction. DMF of lesser purity may be used in the other DMF washes.

STEP 8. *Boc-AA active ester in DMF.*

Use the minimum volume of DMF required to suspend the resin. Use 4 moles of Boc amino acid active ester per mole of the first amino acid on the resin. Greater excesses of active esters and longer coupling times have been used where the coupling reaction was slow. To recover the unreacted excess Boc amino acid active ester from the reaction filtrate, dilute the DMF with water, extract the ester into $CHCl_3$ or EtOAc, and repurify the recovered material by an appropriate method.

STEP 9. *DMF wash (3 times).*

When left overnight, the peptide-resin is given 3 CH_2Cl_2 washes and left in CH_2Cl_2.

Cleavage of the peptide from the resin

Cleavage by HBr-TFA. The apparatus for cleavage of the peptide from the resin using HBr-TFA is shown in Figure 19. Suspend the peptide-resin in trifluoroacetic acid (10 ml per g of resin) in the cleavage vessel. If the peptide contains cysteine, methionine, or tyrosine, in addition to benzyl or carbobenzoxy groups, dissolve 50 moles of anisole or methyl ethyl sulfide in the TFA for each mole of sensitive amino acid (see pp. 9, 21); 15 moles of methionine per mole of sensitive residues has also been found to give satisfactory protection (80). Bubble a slow stream of anhydrous HBr for 90 min. into the suspension by means of a bleeder tube and out through a drying tube containing $CaCl_2$. A shorter cleavage time may be desired (see p. 11). If the peptide contains tyrosine or tryptophan, bubble the HBr through a scrubber tube containing a solution of 2 g of anisole or resorcinol in TFA. Use a trap between the HBr cylinder and the cleavage vessel. The HBr

flow must be adjusted occasionally, since the needle valve often tends to shut itself off, and if it does, the solvent will be sucked back into the tubing. After 90 min., close the HBr tank and clamp off the HBr inlet. Open the stopcock at the base of the cleavage vessel, and aspirate the peptide in the HBr-TFA into an appropriate round-bottom flask. The suction may be applied by means of a water pump. Use a $CaCl_2$ drying tube in the aspirator line to prevent moisture from being sucked back into the flask. Remove the HBr valve after each use, wash with methanol, and dry thoroughly.

Wash the resin 3 times with TFA (10 ml per g of resin each time), allowing the TFA to extract the resin about a minute with each wash, then evaporate the peptide solution to dryness under reduced pressure without heating. Dissolve the peptide several times in a suitable solvent, HOAc–H_2O (3:1) or MeOH–H_2O (1:1), and evaporate the solvent under reduced pressure to remove excess HBr (the peptide is always quite acidic at this stage). If the cleavage solution contained anisole, extract the crude product thoroughly with ether (Sephadex chromatography has also been used for removal of anisole, methionine, and ethyl methyl sulfide), then dry the peptide under high vacuum. The crude peptide from HBr-TFA cleavage always contains much nonpeptide material, so the weight at this point is not a true index of the yield. Yields of crude peptide can be based on amino acid analysis of hydrolysates.

CAUTION: The entire cleavage process should be done in a good hood. Trifluoroacetic acid causes serious burns; give any spillage on the skin immediate attention. *Do not breathe the TFA vapor.*

The use of anhydrous HF in solid phase synthesis (by A. B. Robinson). HF is very easily handled in a Teflon–Kel-F line. However, it can cause severe burns and is quite poisonous. No operation should be performed which can possibly lead to contact with the liquid or vapors. Keep available in the laboratory a 20% solution of calcium gluconate in glycerin.

The commonest HF burns result from accidental contact with HF after which the HF is not completely washed from under the fingernails, and, several hours later, causes a painful burn. The pain can be alleviated by applying calcium gluconate. After contact with HF, wash

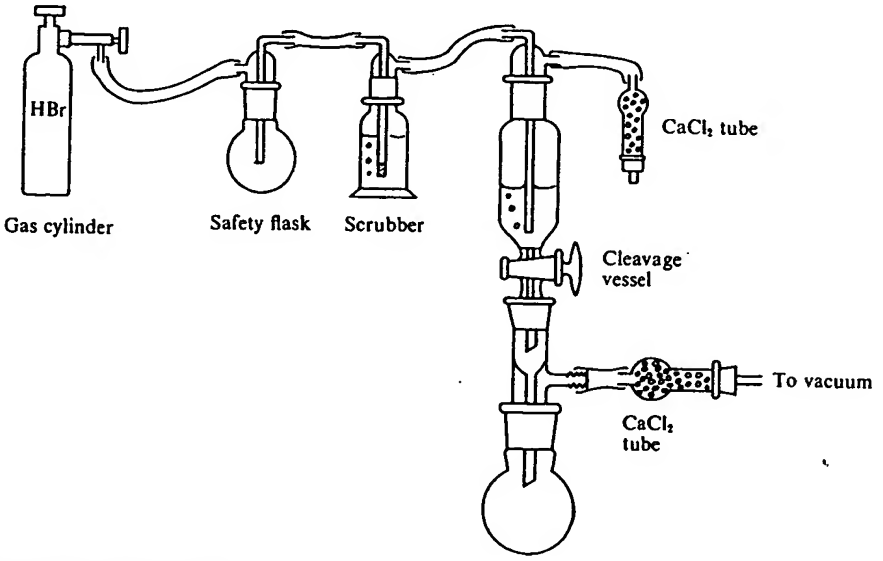

FIGURE 19.
Cleavage of Peptides with HBr-TFA.

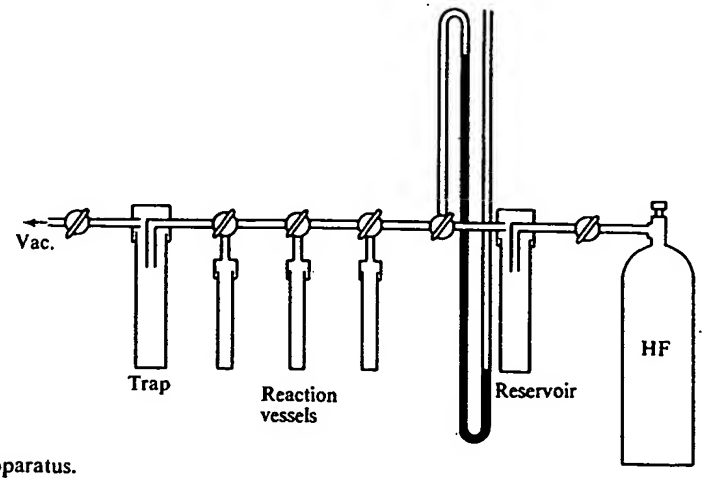

FIGURE 20.
Diagram of HF Apparatus.

liberally with water and soak in water, then apply calcium gluconate.

The entire HF operation *must* be carried out in a good hood: *breathing HF causes death.*

CONSTRUCTION OF AN HF LINE. Figures 20 and 21 show HF lines quite adequate for these reactions (52, 101, 106). The primary requirements for the line are:

1. The materials used must be inert to HF. Teflon and Kel-F are used in the illustrated lines. No metal should come in contact with the HF gas or liquid.

2. The line must be vacuum-tight and also pressure-tight to at least two atmospheres internal pressure.

3. The volumes of HF must be easily measurable. The vessels in the illustrated lines are Kel-F, which is translucent.

The line diagrammed in Figure 20 is commercially available (see Appendix B, item 32).

The line illustrated in Fig. 21 can be constructed in two days with an expenditure of $250 for materials. The valves and connectors are commercially available Teflon pieces, and the rest of the line is machined from Kel-F rod. The flange clamp fittings used in the line-vessel connections are convenient. Viton rubber O-rings are used to seal these connections, and should be changed periodically. (See Appendix B, items 33 and 34.)

A workable HF line has also been assembled from polyethylene tubing and stopcocks and polypropylene tees and screw-cap bottles (97; see Appendix B, items 35, 44, 48, and 49). The tubing was cold-shrunk into undersized holes in the bottle caps, and Teflon tape was used for sealing threads. The total cost of such apparatus would be very much less than for either of those described above, but users should be warned to be especially careful of leaks or vacuum collapse. Dangers arise both from possible admission of water vapor into the system and from HF burns and poisoning. Those who designed this line use a stream of dry N_2, rather than vacuum, to remove HF.

USING THE HF LINE. Any refrigerant of a temperature low enough to condense HF is suitable for its distillation. Liquid nitrogen is the easiest to use, but CO_2-acetone is satisfactory. Although an aspirator will suffice, it is preferable to use a vacuum pump isolated from the line by a liquid nitrogen-cooled trap and a KOH trap. For most convenient operation, use both a vacuum pump and a water aspirator, each connected to the HF line by a 3-way stopcock. Although these reactions seem to proceed satisfactorily with no special precautions about H_2O, it is preferable to dry the HF over CoF_3 in the reservoir vessel. Both the reservoir vessel and the reaction vessel should have Teflon-covered magnetic stirring bars to avoid bumping and to permit distillation at a reasonable

rate. The volume of HF used is measured by calibrating the reservoir vessel and observing the amount distilled from it.

A typical experiment is as follows.

1. Place a stirring bar and a few grams of CoF_3 in the reservoir vessel.

2. Place the dry peptide-resin and a stirring bar in the reaction vessel. Add at least 50 equivalents of anisole per equivalent of peptide if sensitive amino acids are present along with carbobenzoxy, benzyl, or nitro protecting groups.

3. Fill the reservoir and trap Dewars with liquid nitrogen and wait for a few minutes. Kel-F has a low thermal conductivity, and the inside of the vessels will require a few minutes to become safely cold.

4. Open the vacuum-pump valve and evacuate the line. Do not pump long enough to distill the anisole.

5. Close all valves.

6. Slowly open the HF tank valve and distill HF into the reservoir vessel. It is convenient to store enough HF for several reactions in the reservoir.

7. Turn off the HF tank valve and let the reservoir vessel warm to room temperature. A stirred beaker of water will speed heat transfer. Magnetic stirring bars in the plastic vessel and in the water beaker can be stirred simultaneously with the same stirring motor. Continue stirring at a rate which keeps the CoF_3 freely suspended in the liquid HF.

8. Now cool the reaction vessel and slowly open the valve between the reservoir vessel and the reaction vessel so that the HF in the reservoir vessel boils gently. In the presence of anhydrous HF, peptide-resins turn a deep blue. In the presence of anisole and HF, they turn red. When the desired amount of HF has distilled, close all valves and place a stirred bath

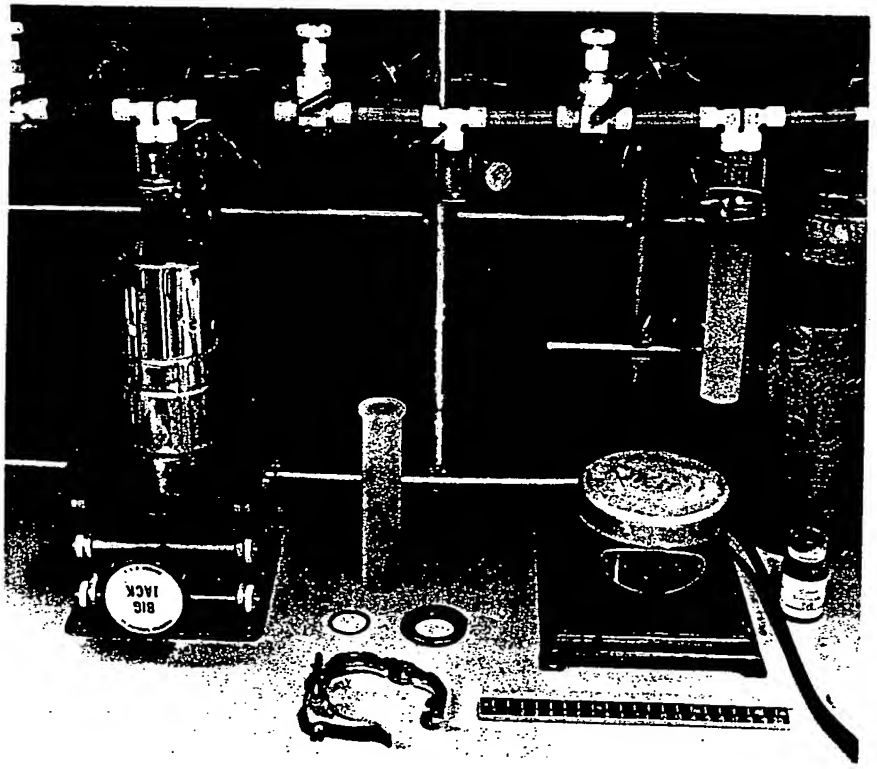

FIGURE 21.
Photograph of HF Apparatus.

of the desired temperature around the reaction vessel. Stir the reaction mixture for the desired length of time.

9. When the reaction is complete, continue stirring and *cautiously* open the valve between the reaction vessel and the trap. Distill all HF into the trap vessel.

10. Throughout steps 6 to 9, the vacuum pump has been isolated from the line. The system should still be under the pressure only of HF. After all the HF has distilled from the reaction vessel, open the line to the pump to effect complete removal of the anisole and the red-orange material. This may take an hour.

11. When the sample is dry, suspend the resin in dry EtOAc and transfer to a peptide cleavage vessel (see Fig. 25). Several washes with EtOAc will remove the last traces of anisole and its oxidation products. Dry the resin *in vacuo* and extract the peptide with a suitable solvent.

a. With peptides of low molecular weight, dissolve the sample in a few ml of 1% HOAc, filter off the resin, and immediately freeze-dry.

b. With large peptides and proteins, immediate desalting on a Sephadex column is preferable. Pyridine or HOAc is useful for extracting water-insoluble peptides from the resin.

12. HF can be most conveniently disposed of by pumping it from the trap vessel directly into the aspirator.

The vessels may be conveniently washed with soap and water, then rinsed with distilled water. Occasionally, the line may be disassembled and boiled in HNO_3. It is very important to keep the HF apparatus *free* of metals, especially with proteins, peptides containing charged side chains, and porphyrins. With proteins, the HF must be dried over CoF_3.

CHEMISTRY. Though we have rationalized mechanisms to direct our experiments, the real mechanisms of the HF reactions here are virtually unknown. Some facts may, however, be helpful.

1. We have used HF on solid phase peptide-resins containing all the common amino acids with good results (101). A typical procedure is 100 micromoles peptide-resin, 0.5 ml anisole (5 millimoles), and 5.0 ml HF, reacted at 0° for 30 min.

2. N–O acyl migration does not appear to be a serious problem. In any case, it can be reversed easily by weak base.

3. The only amino acid which has not, at present, been successfully blocked for Merrifield synthesis and unblocked by HF is histidine. However, see p. 20.

4. Several proteins remain biologically active after long exposure to HF. Cytochromes, however, and other heme proteins lose their Fe completely on contact with HF. If the HF is dry and metal-free, the cytochrome, too, can be returned to biological activity by reinsertion of the Fe. Nonheme iron proteins are similarly bleached.

5. Cysteine peptides are in the free SH form after the freeze-drying, and can be used for further reactions immediately (see p. 48).

6. If O-benzyl tyrosine is present, the reaction time should be at least 1 hour at 0°.

Cleavage by ammonia (*14*, *128*). Saturate anhydrous methanol with anhydrous ammonia at 0°. Add the peptide-resin (10 ml solvent per g of resin) and a magnetic stirring bar, wire or tape a tight stopper in place, and stir the suspension at room temperature for 2 days. Open the vessel, remove the resin by filtration, wash it with methanol, and evaporate the combined filtrate and washings *in vacuo*. For peptides not soluble in methanol, use an appropriate solvent.

CAUTION: The entire operation must be conducted in a good hood.

Somewhat higher yields of some peptides have been obtained by suspending the peptide-resin in purified dioxane, chilling the suspension, adding an equal volume of cold, ammonia-saturated methanol, stoppering, and stirring as above (128). Mixtures of ammonia-saturated methanol and DMF have also been used (44). If ammonolysis of peptides from the resin is unsatisfactory the peptide may be first removed

from the resin as the methyl ester by transesterification, then converted to the amide in solution.

Cleavage by hydrazine (93). Suspend the peptide-resin in purified DMF (5 ml per g of resin) and add anhydrous hydrazine (30 equiv. per equiv. of peptide). Stir the mixture for 2 days at room temperature. Remove the resin by filtration and wash with DMF. Evaporate the combined filtrate and washings *in vacuo*, and purify the peptide hydrazide by a suitable procedure.

Since Boc and formyl amine protecting groups and *t*-butyl esters remain intact during the hydrazinolysis, they can be used to produce protected peptide hydrazides suitable for further coupling in solution. Methyl and benzyl esters on side chains will be converted to hydrazides.

The procedure of Honda *et al.* (37) may be used to prepare anhydrous hydrazine from the hydrate. Add one kg of hydrazine hydrate during 3 hours from a dropping funnel to a gently refluxing mixture of 2 liters toluene and 2 kg CaO. Continue refluxing for 10 hours, then distill the anhydrous hydrazine into a Dean-Stark receiver (see Appendix B, item 24). Remove the hydrazine, which collects as the lower layer, and return the toluene to the flask. The yield, which is about 75%, can be improved on successive runs by reusing the same toluene.

Cleavage by transesterification (56).

METHYL ESTERS. Suspend the peptide-resin in anhydrous methanol (40 ml per g of resin), and add triethyl amine (50 moles per mole of peptide). Stir the mixture at room temperature for 20 hours. Remove the resin by filtration, evaporate the solvent, and purify the peptide ester by a suitable technique.

ETHYL ESTERS. Stir a suspension of peptide-resin in 10% Et_3N–EtOH at 45° for 90 hours. A fair yield of peptide benzyl ester has been similarly obtained by reaction at 80° for 40 hours. Other primary alcohols have been similarly used. B. Halpern has found that transesterification is inhibited in rigorously dried reagents.

Deprotection of finished peptides

Hydrogenation under pressure. The hydrogenation is carried out using a Parr low-pressure, shaker-type hydrogenation apparatus. This procedure includes details for monitoring the reduction of nitroarginine to arginine, but aliquots may also be removed to test for the hydrogenolysis of other protecting groups.

Dissolve the sample to be hydrogenated in MeOH-HOAc-H_2O (10:1:1), usually about 10 to 20 mg per ml, and place in the hydrogenator bottle. Add the catalyst, 5% Pd on $BaSO_4$ (51). For hydrogenolysis of nitroarginine, a weight of catalyst equal to the weight of crude peptide will usually give complete reaction overnight. For hydrogenolysis of benzyl histidine, more catalyst and a longer time may be necessary. Take an 0.1 ml sample for analysis; save for comparison with the aliquot taken after hydrogenation.

INSTRUCTIONS FOR OPERATING THE H_2 TANK. Open the main valve of the H_2 tank. Open the valve to the reservoir of the hydrogenator. With the cylinder needle valve, add H_2 to the reservoir to between 50 and 55 lbs. Close the cylinder needle valve. Close the main H_2 valve, and release pressure in the cylinder needle valve by opening it. Reclose the reservoir valve and the cylinder needle valve.

Place the bottle, with the protector shield on it, in position and evacuate the bottle with a water aspirator. The solvent bubbles at first and then ceases to bubble. Close the valve to the aspirator and fill the bottle with H_2 by opening the valve from the reservoir to the bottle. Close the valve from the reservoir to the bottle, and repeat the evacuation and filling process twice. Leave the valve between the H_2 reservoir and the bottle open during the reduction. Shake overnight. Close the valve to the reservoir and bleed the H_2 from the bottle before loosening the bottle clamp. Remove an 0.1 ml aliquot to

test the completeness of hydrogenolysis, as follows. Add 1 ml MeOH and 9 ml H_2O to the aliquots taken before and after the reduction. For nitroarginine the process of the hydrogenolysis is monitored by observing the disappearance of the ultraviolet absorption at 271 mμ due to nitroarginine (ϵ = 14,970) and the appearance of a positive Sakaguchi reaction (see p. 56) due to the free guanidine group of arginine. For histidine peptides, removal of the benzyl group from the imidazole nucleus is monitored by appearance of a positive Pauly reaction (see p. 56). Electrophoresis or chromatography are also helpful for determining completeness of hydrogenolysis. After the reduction is complete, filter the reduced peptide through a washed $\frac{1}{4}$-inch celite pad and evaporate under reduced pressure (flash evaporator). Dry in a desiccator *in vacuo* over KOH. The peptide is now ready to be purified.

The palladium in the recommended catalyst is present as the brown oxide; it is reduced to the black metal at the beginning of the shaking. Strong acids (HCl) cannot be used, since the PdO will dissolve. Palladium on carbon catalyst can also be used, but is less active. If it is used, the catalyst must be wet first with the water, since the dry catalyst will ignite methanol and other flammable solvents. All hydrogenation catalysts are pyrophoric after the hydrogenation; do not suck air through them on the filter for any extended time.

Hydrogenation at atmospheric pressure. Dissolve the crude peptide either in MeOH with 10% HOAc and 10% H_2O or in 90% HOAc in H_2O in a ⅀ round-bottom flask. Add the catalyst, 5% Pd on $BaSO_4$ (about 1.5 times the weight of peptide), to the flask containing the dissolved peptide. Remove a sample for analysis as described in the preceding section. The apparatus for hydrogenation is shown in Figure 22. Fill the gas-washing bottle with water to prevent excessive evaporation of solvent during the hydrogenation, and allow the operator to estimate the rate of gas flow. Bubble N_2 through the reaction mixture for 10 min. then very slowly bubble H_2 through it for 15 hours at room temperature. Vent the H_2 outlet into the hood flue. Stir the reaction mixture magnetically throughout the operation. Flush N_2 through the line for about 10 min. before opening the vessel. Analyze, filter, and evaporate the reduced peptide to dryness as in the preceding section.

Deprotection by sodium and liquid ammonia. The apparatus shown in Figure 23 is suitable for removing S-benzyl, *im*-benzyl, and guanidino-tosyl groups from simple peptides. The ball joint connecting the two flasks provides flexibility. The ammonia inlet tube should extend nearly to the bottom of the drying flask. Fill the drying tubes with KOH pellets. Assemble the entire apparatus with glass-covered stirring bars in the flasks and silicone grease on the joints. Place the protected peptide in the reaction flask, and pump the entire apparatus overnight on high vacuum, then allow air to enter slowly through the drying tube.

No more than 100 mg of peptide should be reduced at one time; this will require at least 100 ml of ammonia in the reaction vessel. Add a small piece of sodium (freshly cut under dry toluene) to the drying flask. Cool the drying flask in a bath of ethanol and solid CO_2, and condense enough ammonia for the peptide at hand from the cylinder into the drying flask. Excess sodium, as indicated by the blue color, must be present throughout the subsequent distillation of ammonia into the reaction flask.

Transfer the freezing bath to the reaction flask, and distill the ammonia over. Smooth distillation may be aided by stirring. The ammonia in the reaction flask should not be high enough to cover the ammonia inlet tube. When enough ammonia has been condensed in the reaction flask, remove the freezing bath, start the stirrer, and add *small* pieces of sodium, freshly cut under dry toluene and transferred without blotting to the flask with the aid of polyethylene-tipped forceps. All traces of iron must be avoided, since it catalyzes the conver-

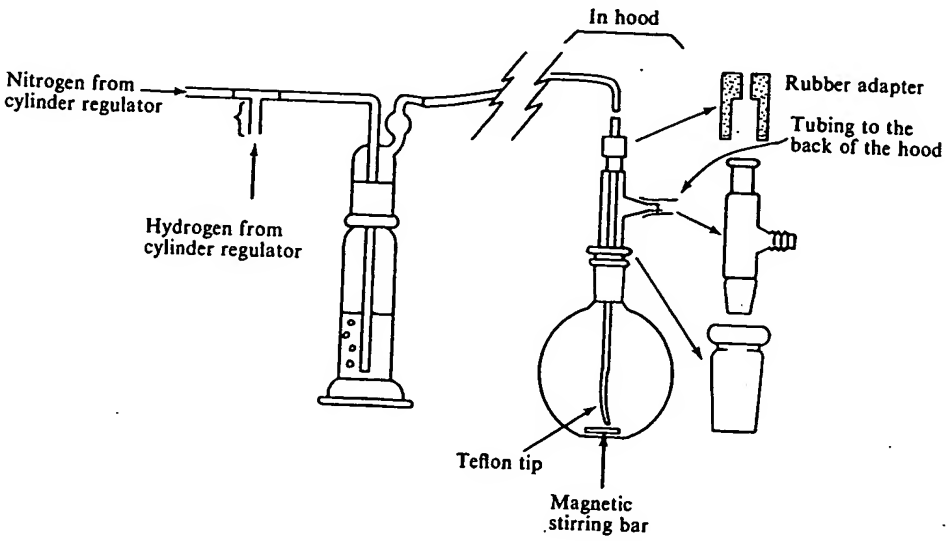

FIGURE 22.
Hydrogenation at Atmospheric Pressure.

sion of Na in NH_3 to $NaNH_2$. Vapors of CO_2 from the bath must not touch the sodium or enter the flask. If the ammonia is boiling during the addition of sodium, the escaping vapors help prevent the entrance of water vapor. Let each piece of sodium dissolve completely before adding another. As the end of the reaction is approached, the pieces of sodium should be very small, so that a large excess of sodium is never present. The end-point is indicated by a stable, very light-blue color in the peptide-ammonia solution. When the blue color has

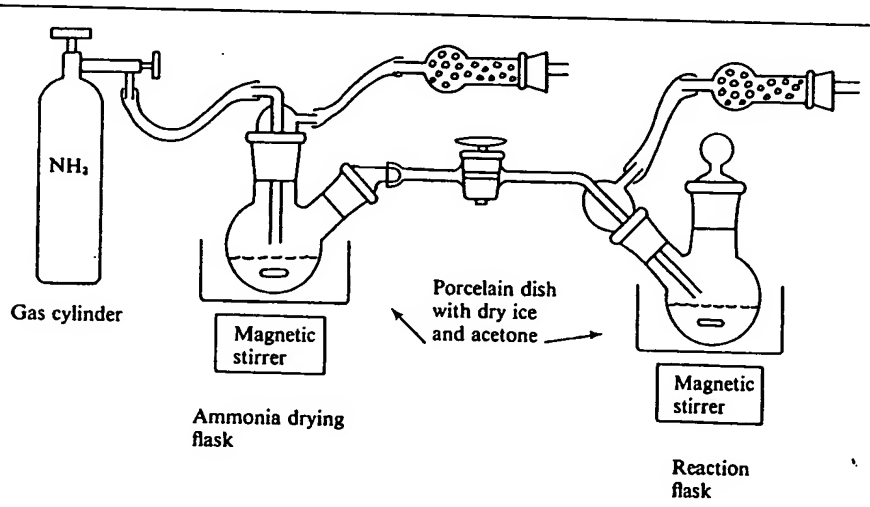

FIGURE 23.
Apparatus for Na–NH_3 Reduction.

been maintained for the desired length of time (see third paragraph below), quench the reaction by adding enough dry ammonium chloride to react with the sodium added (equivalent to the sum of carboxyl, hydroxyl, and deprotected groups).

Let the ammonia evaporate, with stirring (vent into the hood flue). Remove the last traces of ammonia by pumping the flask (through the drying tube) first on the water aspirator, then on a well-trapped vacuum pump. Dissolve the peptide residue in dilute acetic acid or HCl, being careful that the solution does not remain basic any longer than necessary. There is *a danger of explosion* if $NaNH_2$ or unreacted Na is present.

Cysteine-containing peptides can be stabilized for purification in the reduced state by conversion to the S-sulfonate (3, 23). Alternatively, they can be oxidized to the disulfide at *p*H 6.8 by ferricyanide (38), or by iron-catalyzed air oxidation (137).

Much discussion has centered on how long a stable blue end-point should be maintained before quenching the reduction. Early workers used extremely long times (15 to 30 min.). However, recent work with longer peptides has shown that many peptides are seriously degraded by the Na–NH_3 reaction. Thr-Pro and Arg-Pro bonds are especially labile (8, 98). Marglin and Merrifield (62) found that satisfactory yields of the insulin B-chain could be obtained only if the stable blue color was quenched after 15 seconds, and then only if a large excess of sodium was never present. To improve the reduction, Merrifield (77) designed a chilled addition funnel, in which sodium was dissolved in liquid ammonia. This ammonia solution was then added to the reaction vessel in such a way that no large excess was ever present. A similar approach has been used by Bayer *et al.* (5, 6). It is much more difficult to control the amount of excess sodium present when the metal is added directly to the reaction flask. An apparatus for preparing and using Na–NH_3 solutions has been described (89).

Recent evidence indicates that not all peptides can be satisfactorily deblocked by Na–NH_3. A 45-residue peptide which contained two Bzl-His residues and four Pro residues, two in the Thr-Pro linkages, never gave a satisfactory end-point (94). Proline began to degrade before removal of benzyl groups from histidine was complete.

CAUTION: The entire operation must be done in a good hood in a room where ninhydrin reactions are not done. *Metallic sodium* is extremely caustic and *explodes on contact with water*. Ammonia is very caustic to the eyes, and appropriate first aid should be readily available.

Specific techniques with tryptophan residues

Because of the well-known acid lability of tryptophan, the synthesis of tryptophan-containing peptides by the solid phase method has been approached with some hesitancy. However, it appears that, with minor modifications of the standard procedure, tryptophan can be handled satisfactorily in SPPS. The two points in the synthetic procedure which offer a potential hazard are the deprotection of Boc groups and the cleavage of the finished peptide from the resin.

Two apparently satisfactory methods for removing Boc groups from tryptophan-containing peptide-resins are available. M. E. Lombardo and R. Piasio at Schwarz Bioresearch (56) have found that the HCl-HOAc reagent can be used without damage to tryptophan if the temperature is lowered to near the freezing point of the solvent. For this purpose they constructed a jacketed reaction vessel and circulated water at 15° through the jacket during the deprotection and acid-wash steps. At this temperature, deprotection was prolonged to 45 min., and no significant oxidation of tryptophan occurred during the synthesis. In a different approach, G. R. Marshall, at Washington University (64), has incorporated a reducing agent into the acidic media. To the standard HCl-HOAc reagent he added 1% 2-mercapto-

ethanol, then used this modified reagent at room temperature. He also added mercaptoethanol to the HOAc washes after the HCl-HOAc step. Use of mercaptoethanol will cause difficulty if a subsequent hydrogenation step is used. In any event, hydrogenation may be hazardous for tryptophan peptides. Removal of Boc groups with TFA appeared to be less satisfactory.

A logical development of this approach would be the addition of mercaptoethanol to the HCl-dioxane reagent. The thiol should both prevent peroxide formation in the reagent and protect the tryptophan, making the advantages of the dioxane reagent available for use with tryptophan peptides.*

When the Nps protecting group is used in the synthesis of tryptophan-containing peptides, HCl cannot be used for deprotection. The following procedure has been found satisfactory for SPPS (45, 60), and is to be substituted for steps 1, 2, and 3 of schedule A: Step 1—HOAc wash (3 times). Step 2—suspend the resin in MeOH–HOAc (4:1 by volume, 5 ml per g of resin), add thioacetamide (20 moles per mole of Nps groups), and rock for 30 min. Step 3—wash resin with MeOH and HOAc (3 times each).

Anhydrous HF can be used to cleave tryptophan-containing peptides from the resin without loss of any tryptophan (64). This was to be expected, since Sakakibara had already shown that HF does not damage tryptophan (106). Cleavage with HBr-TFA caused at least a 15% loss of tryptophan. As alternate methods of cleavage, any of the basic reagents can be used. Ammonolysis (64), transesterification with an alcohol and triethyl amine (56), and sodium ethoxide cleavage (55) have all been used successfully for tryptophan-containing peptides.

The amino acid analysis of tryptophan in peptides presents a special problem, since the usual acid hydrolysis cannot be used. The usual approach to this problem has been to use alkaline hydrolysis (86, 99). Hydrolyze peptides in sealed, evacuated Pyrex tubes with 12% NaOH for 22 hours at 110°. Acidify the hydrolyzate to *p*H 2 with HCl, and remove by filtration the silicic acid resulting from attack on the glass by alkali. Dilute the sample to a sodium concentration of no greater than 0.2 *M* before applying it to the amino acid analyzer. An approach which avoids the formation of silicic acid uses screw-capped Teflon tubes, and hydrolysis with 15% NaOH for 16 hours at 110° (90), although it is sometimes hard to get a good seal. Marshall (64) found that the most reliable and consistent results with tryptophan peptides could be obtained by complete enzymatic hydrolysis of them. A new method for rapid enzymatic total hydrolysis has been developed by Keutmann and Potts (46; see p. 54). Their method uses successive treatments with large amounts of papain and aminopeptidase M, and appears to be free of the difficulties encountered with leucineaminopeptidase, which failed to cleave Arg-Pro and Asp-X bonds. A spectrophotometric method for measuring tryptophan based on loss of UV absorption after treatment with N-bromosuccinimide has been described (125).

*E. Kaiser and R. Colescott have recently used such a reagent successfully for the synthesis of tryptophan peptides.

TECHNIQUES FOR PURIFICATION OF PEPTIDES

Purification by countercurrent distribution

Countercurrent distribution has been used to purify many peptides synthesized by solid phase. Although long CCD runs are required for very high resolution, most peptides prepared by the solid phase method, especially ones of moderate size, are fairly pure as cleaved from the resin, and short CCD runs will provide the necessary purification. Runs of 100 to 300 transfers are usually adequate. CCD has the advantages that fairly large amounts of material can be handled with much greater ease than they can be with

column chromatography, and that purification by CCD usually takes less time. (For details of the procedure, see 20, 21, 34.)

A particular problem with CCD that must be kept in mind is selection of a solvent system which will provide a satisfactory k without causing degradation of the peptide or intractable emulsification, and from which the product can be recovered without too much difficulty. Salt-containing systems, which have been so useful for proteins, cause difficulty with small peptides, since dialysis cannot be used to separate the peptide from the salt. In general, where buffering is desired, volatile systems using ammonia or pyridine with acetic or formic acid have proved most satisfactory. Moderate amounts of salts may be removed by gel filtration on Sephadex columns, but inconveniently large columns are required if the salt concentration is high.

After completion of the CCD run, peptide peaks are located by applying a quantitative reagent (see pp. 55–57; see also the section after this one).

Purity of peptides can be demonstrated by analysis of aliquots from both upper and lower phases of each tube across the peak; the k will be constant across the peak if the substance is pure. The shape of the peak should also conform to the theoretical curve. For more rapid location of peaks when purity is to be established by some other criteria, one need sample only the lower phase of the first half of the train and the upper phase of the second half.

Aliquots for analysis should be as small as is compatible with accurate analysis, since large samples of upper phase may not be miscible with the analytical reagents. In butanol systems, aliquots of 0.05 to 0.2 ml are usually satisfactory, whereas larger ones will have to be evaporated.

The following are some systems which have been found useful (solvent ratios are by volume):

1-butanol, 1% trifluoroacetic acid (bradykinin, k 1.2);

1-butanol, 0.4 M ammonium acetate, pH 7 (angiotensin I, k 0.8; angiotensin II, k 0.13);

1-butanol, acetic acid, water, 4:1:5 (angiotensin II, k 0.3);

1-butanol, 2% formic acid;

1-butanol, pyridine, acetic acid, water, 150:2:0.2:150 (or 8:2:1:9);

1-butanol, benzene, TFA, water, 135:15:3:150 (bradykininyl-bradykinin, k 2);

1-butanol, pyridine, 0.1% acetic acid, 790:475:1735 (insulin A- and B-chain S-sulfonates);

0.086% trichloroacetic acid in 2-butanol, water, propionic acid, 87:110:15, the TCA being extracted from the purified material with ether (Bence-Jones protein, k 0.5);

88% phenol, ethanol, 0.1 N HCl, 1750:895:2510 (hemoglobin tryptic peptides); and

acetic acid, water, 2-butanol, cyclohexane, pyridine, 30:70:60:40:2 (tyrocidin C, k 0.8).

Column chromatography

Ion-exchange column chromatography is the most widely used technique for separating and purifying peptides because of its great versatility and extreme selectivity. Significant amounts of peptides can easily be handled in a single run (though not as much as with CCD), even though a single run sometimes takes a long time. A brief guide to some different types of chromatography is presented in this section. Ion exchangers based on cellulose have been superior for large peptides and proteins. General information is available in recent books (2, 36). Systems useful with peptides all use volatile buffers.

Chromatography on Dowex 1 (28). Dowex 1 is a strongly basic resin composed of quaternary ammonium groups on a crosslinked polystyrene matrix. The system of Funatsu (28) allows the entire range of peptides, from strongly basic to strongly acidic, to be chromatographed in a single run on this resin. For separation of such a complete mixture, the sample is adsorbed to the acetate form of the resin at high pH (8.8,

pyridine-collidine). The peptides are eluted, first with a gradient of decreasing *p*H pyridine-collidine-acetate buffers, and then with a gradient of increasing concentration of acetic acid. The entire system takes 3 days, but for the purification of synthetic peptides, only a short part of the entire system will generally need to be used. Basic peptides (especially those containing much arginine) are eluted first, followed by the neutral, and then the acidic peptides. Peptides containing a high percentage of aromatic amino acids will be retarded more than would be expected from their net charge.

Dowex 1 × 2 resin is converted to the acetate form by cycling batchwise with 1 *N* NaOH, 1 *N* HCl, 1 *N* NaOH, and 50% HOAc using H_2O washes after each cycle. A 0.9 by 150 cm column of this resin, operated at 40°, will handle up to 30 micromoles (100 mg) of a peptide mixture, somewhat less if the sample is largely one peptide. The solvent gradients are established with a Varigrad, and are pumped through the column by a metering pump. (For details of solvent preparation, see 28.)

After the end of the run (50% HOAc elution), the column can be prepared for reuse without repouring by washing it with water and then equilibrating it with the starting buffer by pumping it overnight or until the effluent reaches the proper *p*H.

Chromatography on Dowex 50 (112). Dowex 50 is a strongly acidic resin bearing sulfonic acid groups on a crosslinked polystyrene matrix. The procedure of Schroeder (112) allows a wide range of peptides to be separated on this resin by buffers of volatile pyridine in acetic acid. One hazard in the use of Dowex 50 is that peptides with a high proportion of aromatic amino acids may be irreversibly bound to the resin.

Dowex 50 is cycled with NaOH and HCl, and converted to the pyridine salt with 2 *M* pyridine. The resin is equilibrated in a *p*H 3.1 buffer of pyridine in acetic acid (0.2 *M* in pyridine), and the column is run at 38° with a gradient from the equilibrating buffer to *p*H 5.0, 2 *M* pyridine buffer, and then with a *p*H 5.6, 8.5 *M* pyridine buffer. (For details of buffer preparation and operation, see 112.)

Chromatography on IRC-50 (25, 72). IRC-50 is a weak acid resin, bearing carboxyl groups. It is especially useful with basic peptides; aromatic residues do not have any great affinity for it, and acidic peptides may not be held on columns of it. IRC-50 is cycled with acid and base, and converted to the free-acid form by washing with 50% HOAc. When cycled with acid or base, the resin does not neutralize instantaneously, as the strong acid and strong base resins do, and must be allowed to stand with the reagent for about 30 min.

Columns of IRC-50 can be run in two different ways. Peptides can be adsorbed to the hydrogen form of the column from solution in water and eluted with a gradient of increasing strength of acetic acid (72). A basic peptide, such as bradykinin, will elute from the column with approximately 35% HOAc. Higher resolving power is obtained by eluting the peptides with ammonium acetate or pyridine acetate buffers of increasing *p*H, or by increasing ionic strength at constant *p*H (25). In this type of operation, bradykinin will be eluted at approximately *p*H 6.8, with a 0.125 *M* ammonium acetate buffer.

Gel chromatography. Chromatography on dextran or polyacrylamide gels is a very useful technique for separating peptides from salt and other contaminants of low molecular weight, and for separating peptides on the basis of molecular weight. A good recent source for both general and specific information on gel chromatography is H. Determann, *Gel Chromatography* (Springer, 1968).

Analysis of effluent fractions. Peptides can be located in the eluates by the quantitative

ninhydrin or Folin-Lowry reactions, or by one of the reagents for specific amino acid residues (see pp. 56–57). The Folin-Lowry method is more sensitive, especially for large peptides. Sensitivity of the ninhydrin reaction with peptides can be greatly increased by a preliminary rapid alkaline hydrolysis (35). If the ninhydrin reaction is to be used to analyze fractions from columns developed in pyridine or collidine buffers, these solvents must be redistilled from a flask containing some ninhydrin to remove contaminants which react with it. If the Folin-Lowry reaction is to be used on fractions in pyridine or collidine buffers, the samples must be evaporated to dryness in order to remove pyridine and collidine, which interfere. This evaporation can be conveniently done by pipetting aliquots from the eluate fractions into test tubes, assembling the tubes into a vacuum desiccator, and pumping under high vacuum. Aliquots of 0.05 ml should be dry in about an hour, 0.1 ml aliquots in 4 hours. The ninhydrin, Folin-Lowry, Sakaguchi, and Pauly tests are all *p*H-sensitive, and due attention must be given to neutralization of excess acid or base.

Preparative paper chromatography

Although paper chromatography is most commonly used to ascertain the purity of peptides, it can be used to purify small amounts of peptides (1 to 50 mg or micromoles). If the peptide is found to be heterogeneous by paper chromatography, the solvent which has revealed its heterogeneity can be used to purify it.

SOLVENTS:

Solvents F, I, and M in Table 3 (p. 59) have often been used in preparative peptide separations. Pyridine may be purified as described by McDowell and Smith (68).

PROCEDURE FOR DEVELOPMENT:

The paper used for separating up to 15 mg (15 micromoles) of peptide is usually Whatman no. 1 or no. 4. Whatman 3 MM is used to separate larger quantities (up to 50 mg). The paper must be washed by overnight descending chromatography in the solvent to be used, and dried before use. Descending chromatography is preferable to ascending chromatography for preparative work, since longer times may be used and the resolution is superior. If the solvent is to be allowed to run off the sheet, serrate the bottom for more even dripping of solvent from the sheet. Apply the sample (which must be relatively salt-free) along the origin in 2 to 5 μl amounts per application and dry with a hairdrier before respotting. Put a spot of the peptide to be purified at either edge of the chromatogram as a marker. Some peptides stick to dry paper. If they are applied to paper and dried completely, they do not migrate, or migrate only partially from the origin. Such peptides are spotted in the chamber after the developing solvent has already wet the origin, or are applied to the paper and not completely dried before development with solvent.

LOCATION AND ELUTION OF PEPTIDES:

After solvent development, remove the sheets, using plastic gloves, and air-dry them. Cut off the marker strips, and stain them for location of the bands (using one or more of the sprays listed on pp. 62–64). Cut the areas of paper bearing the desired peptides from the sheets and elute them. The eluting solvent is usually H_2O, 0.1 *N* NH_3, or 1 *N* HOAc. Either extract the strips with the eluting solvent or lay them onto wet, prewashed Whatman 3 MM paper wicks in chromatographic troughs, and elute the samples from the strips by descending chromatography overnight into beakers in an enclosed chamber. Check the completeness of the elution of the strips by an appropriate identifying reagent. The eluates, after filtration, are concentrated *in vacuo* or lyophilized.

ANALYTICAL TECHNIQUES

Amino acid analysis

Hydrolysis of peptide-resins and aminoacyl-resins. Transfer the resin to which the peptide or amino acid has been coupled to a tared 50- or 100-ml ⅄ 24/40 round-bottom flask. After reweighing, record the weight of the resin. For amino acid analyzers using 0.5 micromole samples, about 10 mg of resin is usually adequate. If the resin is not dry, as when portions of resin are removed after each coupling reaction, connect the flask to a high-vacuum pump using the ⅄ 24/40 connectors (Appendix B, item 30) and dry it to constant weight. Add to the flask 5 ml of peroxide-free dioxane and 5 ml of concentrated HCl. Fit the flask with an air condenser and, after refluxing overnight on a hot plate set at low heat, filter the hydrolysate quantitatively through a porcelain Buchner funnel containing a 1.5-cm Whatman no. 1 filter-paper disc directly into another 100-ml round-bottom flask by using an adapter (Appendix B, item 8). Evaporate the filtered hydrolysate to dryness on a rotary evaporator under reduced pressure. Some nonvolatile liquid residue will remain because of hydrolysis of part of the dioxane, but it will not interfere with the amino acid analysis. If the peptide contains Glu or Gln, rehydrolyze the hydrolysate for 24 hours using 10 ml of 6 *N* HCl in order to obtain a proper analysis for this amino acid. These amino acids form esters with the alcohols produced by partial hydrolysis of dioxane. In the 50% dioxane medium, the equilibrium point of this esterification favors ester formation rather than hydrolysis. (Even on rehydrolysis the glutamic acid value may sometimes still be lower than that obtained after cleavage from the resin.) If a second hydrolysis is used, again remove the HCl by rotary evaporation. Add several aliquots of water to the hydrolysate and evaporate in order to remove most of the HCl. Then dilute the sample appropriately with the *p*H 2.2 diluting buffer used for amino acid analysis.

Hydrolysis of peptides. Peptides are hydrolyzed by 6 *N* HCl, after cleavage from the resin, in the usual way, either in sealed tubes (22 hours at 110°) or in 50-ml ⅄ 24/40 round-bottom flasks on a hot plate set at low heat (refluxed overnight). Using the latter technique, the final volume of the sample during hydrolysis should be at least 10 ml, since the peptide may be charred if the volume is too low.

Nitroarginine, on hydrolysis, yields arginine, ornithine, and NO_2-arginine, and the arginine content is calculated from the sum of these three amino acids. On the Spinco 120B Amino Acid Analyzer equipped with the AA15 and AA35 resins, ornithine appears at the position of lysine on the short column and nitroarginine at the buffer change from first buffer to second buffer on the long column. If only NO_2-arginine is on the resin, all 3 products can be satisfactorily determined by a single short-column (basic amino acids) run, taking the neutral peak as NO_2-arginine and adding it to the ornithine and arginine. In lieu of determining actual constants by using NO_2-Arg and Orn standards, the constant for NO_2-Arg may be taken as 0.866 times the average constant of the analyzer and that for Orn as 1.14 times the average constant. These and other amino acid derivatives, with their respective constants and times of emergence from the amino acid analyzer, are given in Table 1.

Certain amino acids may undergo serious loss during hydrolysis. In peptides containing both tyrosine and nitroarginine, mutual loss of these residues may occur. The low value obtained for glutamic acid in dioxane-HCl hydrolysates of peptide-resins has already been mentioned. Amino acids which are susceptible to oxidation (serine, threonine, tyrosine, cysteine, methionine) may be partially destroyed during hydrolysis. Peptides containing such residues should be hydrolyzed in evacuated sealed tubes.

The evacuation is done as follows. Place the peptide, dissolved in 6 *N* HCl (1 to 3 ml), in a 6-inch test tube. Soften the tube near the mouth in a flame and draw it out so that a narrow

TABLE 1.
Analysis of some common amino acid derivatives.[a]

Compound	Constant	Emergence time
Long column for acidic and neutral amino acids (*p*H 3.28 to 4.25):		
Cysteic acid	20.5	18 min.
Met sulfoxide	20.3	42 min. (just before Asp)
Nitro-Arg	18.5	138 min. (near buffer change)
O-Me-Tyr	17.5	35 min. after Phe
S-Bzl-Cys	16.3	45 min. after Phe
Tos-Arg	15.2	75 min. after Phe
Short column for basic amino acids (*p*H 5.28):		
Dnp-His	–	14 min.
ε-Tos-Lys	28.5	17.5 min.
Orn	24.4	18 min. (with lysine)
Trp	17.5	20 min.
im-Bzl-His	19.5	10 min. after buffer change[b]

[a] This table is for a Spinco Model 120B Amino Acid Analyzer, equipped for 4-hour analysis of protein hydrolysates. The average constant for this instrument is 21.37. The following formula may be used to estimate constants for these derivatives on another analyzer (B):

$$\text{Constant on analyzer B} = \left(\frac{\text{constant from Table 1}}{21.37}\right) \times \text{(average constant of analyzer B).}$$

[b] For Bzl-His, the short column is changed at 20 min. to a *p*H 7.0, 0.38 *M* buffer.

constriction is formed. Freeze the sample in a bath of Dry Ice and propanol. Connect the tube to a well-trapped vacuum pump and evacuate. Cautiously allow the frozen sample to warm just until all the ice is melted. During the melting, swirl the tube gently, holding it with the fingers just above the solution level (the warmth prevents a solvent bubble from rising up the tube), and hold the hose to the vacuum system so that it can be closed off instantaneously if bubbling gets out of control. Refreeze the sample and seal off the tube at the constriction, still under vacuum.

Sensitive amino acids can sometimes be protected by adding 0.1% phenol or mercaptoethanol to the acid before hydrolysis (109).

Tryptophan is usually completely destroyed by acid hydrolysis, and other methods of hydrolysis must be used, such as alkaline hydrolysis (see p. 49) or the enzymatic method described next.

Total enzymic hydrolysis using papain and aminopeptidase M (46).

REAGENTS:

1. Buffer for papain incubation—0.05 *M* ammonium acetate, *p*H 5.3.
2. Papain, 0.5 mg per ml, in H_2O.
3. Mercaptoethanol, 1:32 dilution in H_2O.
4. Buffer for Aminopeptidase M incubation—0.2 *M* trimethylamine acetate, *p*H 8.2.
5. Aminopeptidase M (APM) 10.0 mg per ml, in H_2O.

PROCEDURE:

Dry approximately 0.1 mg of polypeptide substrate in the bottom of a conical centrifuge tube. Add 85 μl of reagent 1 and 5 μl of reagent 3, giving a mercaptoethanol concentration of 0.02 *M* during digestion. Add 10 μl of reagent 2 (5 μg of papain) to the buffered substrate, providing a papain con-

centration of 50 μg per ml (enzyme-substrate ratio, 1:20, wt for wt) in a volume of 100 μl. Incubate the mixture at 37° for 2 hours, then inactivate the papain with 2 drops of HOAc and lyophilize the solution. Lyophilization is complete in 30 to 60 min. because of the small volume.

Next dissolve the partially digested mixture in 80 μl of reagent 4 and 5 μl of reagent 3. Add 15 μl of reagent 5 (150 μg of APM), giving a concentration of 1.5 mg per ml of APM (enzyme-substrate ratio, 1.5:1, wt for wt), again in a volume of 100 μl. Carry out the APM digestion for 3 hours, lower the *p*H with HOAc, and lyophilize the mixture. It may then be taken up directly in the appropriate volume of amino acid analyzer buffer for application to the column.

For polypeptides larger than 80 to 90 residues (such as ribonuclease), it is necessary to use a larger APM concentration for a somewhat longer time. Thus, 300 μg of APM are used for 3 hours, then a second 300 μg aliquot is added, and the incubation carried out three more hours; yields are then as satisfactory as those obtained with the smaller peptides.

Amino acids from autodigestion of the enzymes (determined in separate enzyme blanks) amount to less than 0.002 micromoles (and less than 0.0005 micromoles for most residues). In the presence of substrate, autodigestion is even less.

The method has been shown to effect quantitative recovery of asparagine, glutamine, tryptophan, cystine, trifluoroacetyl lysine, mono- and di-iodotyrosine, and nitroarginine, which are normally altered in acid hydrolysis. In the presence of the mercaptoethanol concentrations used, methionine and methionine sulfoxide are retained in their original state.

Chloride analysis by the modified Volhard method (33)

PRINCIPLE:

The sample is acidified with HNO_3 and the chloride is precipitated with a measured excess of standard $AgNO_3$ solution. The AgCl that is formed is coated with toluene and the excess $AgNO_3$ is back-titrated with standard NH_4SCN solution, using ferric alum ($FeNH_4(SO_4)_2 \cdot 12H_2O$) as an indicator. A red color, due to the formation of $Fe(SCN)_3$, indicates that an excess of SCN^- is present and that the end point has been reached.

REAGENTS:

1. Standard $AgNO_3$ (0.1 *N* = 16.989 g per l).
2. Standard NH_4SCN (0.1 *N* = 7.612 g per l). Since the NH_4SCN must be standardized against $AgNO_3$, so that 1.0 ml of NH_4SCN equals 1.0 ml of standard $AgNO_3$, prepare a slightly more concentrated solution of NH_4SCN and dilute it to 0.1 *N* after titration. The titration procedure is the same as described below, except that no sample is added. Calculate the amount of H_2O that should be added to the NH_4SCN to make 10 ml of the NH_4SCN exactly equivalent to 10 ml of $AgNO_3$. Repeat the procedure in the presence of a known concentration of NaCl (1 ml of 1 *N* NaCl) to check the test.
3. NaCl (1 *N* = 58.45 g per l).
4. Saturated ferric alum (124 g of $NH_4Fe(SO_4)_2 \cdot 12H_2O$ in 100 ml H_2O).
5. Toluene.
6. HNO_3 (1 *N*).

PROCEDURE:

Pipet a sample of standard NaCl solution into about 10 ml of H_2O in a 250-ml Erlenmeyer flask and protect from bright light. For titration of HCl-HOAc, use a 1 ml sample. To the flask add about 3 drops of reagent 4 and 1 ml of 1 *N* HNO_3. Put a magnetic stirring bar in the flask and place it on a magnetic stirrer. Slowly add 20 ml of standard $AgNO_3$, with stirring. Stop the stirrer and let the mixture stand for 5 min. Add about 50 ml of water, followed by toluene, so that about a $\frac{1}{4}$-inch layer of toluene is made on the water surface. Mix well with the stirrer. With the stirrer on, titrate with standard

NH_4SCN solution. The first permanent tinge of red-brown indicates the end-point.

CALCULATION:

For HCl-HOAc, [20 − (ml of NH_4SCN)] × 0.1 = *N* of HCl in HOAc.

When the Volhard method is used to determine chloride in solutions containing organic solvents other than acetic acid, results are better if the sample is diluted with 50 ml of HOAc before any reagents are added (77).

Quantitative Sakaguchi determination of arginine (138)

The Sakaguchi reaction gives a red color from unsubstituted or monosubstituted guanidines; more highly substituted guanidines do not react. Therefore arginine and arginine peptides give a positive reaction, while blocked arginines (e.g., nitro, tosyl) and their peptides are negative. The reaction is useful for following the hydrogenolysis of nitroarginine and for locating arginine peptides in CCD and chromatography.

SOLUTIONS:

1. Unknown: samples of peptide solutions or aliquots taken before and after hydrogenation of peptide, diluted with MeOH-H_2O (1:9) to contain 0.02 to 0.1 micromole of Arg per ml.
2. Standard: arginine, 0.05 micromole per ml (8.70 mg per liter); use 0.1 to 2.0 ml, and make up to 2.0 ml with water.

REAGENTS:

1. Dissolve 1 mg of α-naphthol per ml of MeOH, then dilute 1:5 with water, to give 0.02% α-naphthol in 20% MeOH (good for about 2 months).
2. A 2.5 *N* solution of NaOH.
3. NaOBr, prepared by dissolving 0.67 ml of Br_2 in 100 ml of 1 *N* NaOH (good for 2 weeks).
4. A 40% solution of urea in H_2O.

PROCEDURE:

To 2 ml of the standard or unknown solution, add 0.4 ml of reagent 2 (an acidic sample may need more NaOH; the solution must be strongly basic) plus 0.4 ml of reagent 1. Mix and chill in an ice bath. Add 0.04 ml of reagent 3; mix and immediately add 0.4 ml of reagent 4. Mix and read at 515 mμ (optimum color is obtained 20 seconds after addition of reagent 3). Although 5 μg of arginine hydrochloride gives an absorbance of 0.2, the molar extinction coefficient obtained from arginine in peptides may be much lower than that of free arginine. For example, the color yield of bradykinin is only about 70% of that expected, and some proteins have been reported to give color yields as low as 20%.

Quantitative Pauly determination of histidine and tyrosine

Histidine and tyrosine, either free or in peptide linkage, couple with diazotized sulfanilic acid to give a red product. This reagent is particularly useful for following the deprotection of the imidazole nucleus of histidine, since blocked imidazoles do not react.

REAGENTS:

1. Sodium nitrite, 5% in H_2O.
2. Sulfanilic acid, 0.5% in dilute HCl (5 ml concentrated HCl + 95 ml H_2O). (Store these two reagents in the cold, and use while cold.)
3. Sodium carbonate, 10% in H_2O.

PROCEDURE:

Mix in the cold equal parts of reagents 1 and 2, and let stand at least 5 min. To 1 ml of sample (must not be strongly acidic or basic), add 1 ml of reagent 3 and 1 ml of the mixed reagents. Read immediately at 500 mμ.

If alcohol is present in the sample it causes a yellow background which interferes, and better results can be obtained by reading at 520 mμ, although the values are lower.

Ammonia also interferes, and must be removed by alkaline evaporation.

When read immediately, 2 μg of histidine dihydrochloride or 10 μg of tyrosine gives an absorbance of 0.1 at 500 mμ. Color develops to a maximum 30 seconds after addition of the diazotized sulfanilic acid, fades in 3 min. to approximately half the density, then is stable for 15 min.

Determination of peptides by the Folin-Lowry method (58)

The Folin-Lowry color reaction is very useful for determining the presence of peptides. It is most sensitive for long peptides containing phenolic groups, but small nonaromatic peptides can also be detected. It has the advantage over ninhydrin of being easier to prepare, not requiring a heating step, and NH_3 does not interfere. Pyridine and collidine do interfere, and if present must be removed by drying the sample. The *p*H of the sample must be close to neutrality. The color curve is not linear; for accurate determinations of concentrations, a standard curve must be made (see Table 2).

REAGENTS:

1. Na_2CO_3, 2% in 0.1 *N* NaOH.
2. $CuSO_4 \cdot 5H_2O$, 1% in H_2O.
3. Sodium potassium tartrate, 2% in H_2O.
4. Combine 0.5 ml of reagent 2 and 0.5 ml of reagent 3, and make up to 50 ml with reagent 1. Make up fresh each day.
5. Dilute commercially available Folin-Ciocalteau phenol reagent (1:1) with water (it should then be 1 *N* in HCl).

PROCEDURE:

To between 0.05 and 0.2 ml of the sample, add 3 ml of reagent 4 and let stand 10 min. Run a reagent blank at the same time. Add 0.3 ml reagent 5, with shaking. After 30 min. read the color at 700 mμ. Subtract the reagent blank. The volumes can be modified as long as the proportion of reagent 4 to reagent 5 is 10:1. If the sample volume is too high, the volume of the reaction mixture can be reduced by preparing reagent 4 at double strength and using 1.5 ml of it to 0.3 ml of reagent 5, or by decreasing the volumes of the normal reagents 4 and 5 to 1.0 and 0.1 ml, respectively.

The quantitative ninhydrin reaction (87)

The ninhydrin assay for quantitative estimation of peptides is useful for small peptides, for peptides containing a high percentage of lysine

TABLE 2.
Representative color values from the Folin-Lowry reaction.

Material	Amount of material and final volume of Folin reagent	Absorbance at 700 mμ	Absorbance for 1 mg in 1 ml of Folin reagent
Tobacco mosaic virus protein, 18 mg per micromole	0.04 mg in 2.4 ml	0.343	22.6
TMV protein tryptic peptide 8 (20 amino acids), 2.2 mg per micromole	0.01 micromole in 1.1 ml	0.567	31.1
Leu-Asp-Ala-Thr-Arg, 0.57 mg per micromole	0.1 micromole in 1.1 ml	0.71	13.7

residues, or for large peptides after a preliminary rapid alkaline hydrolysis (35). To carry out this hydrolysis, evaporate aliquots to dryness in polypropylene test tubes in an oven. To each tube add 0.15 ml of 13.5 *N* NaOH, and autoclave the tubes for 20 min. at 15 psi. Neutralize the alkali in the cooled tubes by adding 0.25 ml of HOAc to each one.

If the ninhydrin analysis will be used for samples in pyridine-containing buffers, the pyridine must be distilled from ninhydrin before use. If samples in ammonia buffers are to be used, the samples must be made strongly alkaline with NaOH and evacuated in a desiccator for some time (or evaporated to dryness in an oven) to remove the ammonia, and then neutralized with an equivalent amount of acetic acid before analysis. Samples must not contain more acid or base than can be neutralized to *p*H 5 by 1 ml of 0.1 *N* base or acid, otherwise the color formation will be inhibited.

REAGENTS:

1. Sodium acetate buffer, *p*H 5.5, 4 *M*. Dissolve 2720 g of $NaOAc \cdot 3H_2O$ in 3 liters of water by warming. Cool to 25°, add 500 ml HOAc, and make up to 5 liters with water. Adjust *p*H to 5.51. Store at 4° without preservative.
2. Ninhydrin reagent. Dissolve 20 g ninhydrin and 3 g hydrindantin in 750 ml of peroxide-free methyl cellosolve (cellosolve should not give more than a light straw color when mixed 2 to 1 with 4% KI solution). Do not shake or stir vigorously, or the hydrindantin will be oxidized by the air. Add 250 ml of reagent 1, bubble nitrogen through the reagent for several minutes, and store in a dark bottle under nitrogen. The reagent is good for about a week. Many investigators find it more convenient to make up each time just the amount needed, without bothering with the nitrogen aeration and storage. For greater convenience, a table can be made, showing the amounts of the different components needed for various quantities of reagent.

PROCEDURE:

For 1- or 2-ml fractions, use 1 ml of reagent 2. Do not neutralize if less than 1 ml of 0.1 *N* NaOH or HCl will give *p*H 4-6. After addition of the reagent, mix gently by swirling, and cap tubes loosely. Heat for 15 min. in a boiling water bath, and cool immediately. Add 5 ml of 50% EtOH to each tube, and shake well to air-oxidize the remaining hydrindantin (this gives a more stable baseline). Tubes are read at 570 mμ, except for N-terminal proline or hydroxyproline peptides, which are read at 440 mμ, or at the absorption maximum, if different. The standard is leucine, at 50 μg per ml; 0.2 ml of this solution gives an absorbance of 0.2.

Paper and thin-layer chromatography

Paper chromatography (*123*). Paper chromatography, although not so rapid as TLC, is very useful for characterizing and estimating the purity of peptides. Chromatography may be done by either the ascending or the descending method; Whatman no. 1 paper is generally used. Some of the solvents which are commonly used for peptides are given in Table 3. Strongly acidic (formic acid) and basic systems should be used with caution, since they may cause degradation of some peptides. (For identification sprays, see pp. 62–64.) The relative mobilities of peptides in several of the solvents are given in the subsection on TLC of peptides.

Thin-layer chromatography (*49*, *96*, *126*). Thin-layer chromatography is useful for estimating the purity both of starting materials for SPPS and of synthesized peptides. TLC plates may be spread using Brinkman Silica Gel G or Silica Gel H; both give essentially the same Rf's. Precoated glass plates, coated plastic sheets, plastic sheets or glass plates coated with cellulose powder, and other media and equip-

ment for TLC are commercially available.

Plastic sheets are laid against glass plates to hold them erect during solvent development. An advantage of the sheets is that they may be cut after chromatography so that several identifying sprays can easily be used. For chromatographing one or two spots, they can be cut into narrow strips and developed in large test tubes.

The solvents which have been useful for TLC of amino acid derivatives and peptides are given in Table 3. The atmosphere in the developing chamber must be fully saturated with the solvent, or Rf's will not be reproducible. Sheets of filter paper moistened with the solvent and stuck to the walls of the chamber help saturate the atmosphere. Standards should always be run along with unknowns if possible.

TLC OF AMINO ACID DERIVATIVES. The amino acid derivatives used in SPPS must be of high purity. Every order received, even of the same lot number, should be checked for its purity by TLC.

Spot approximately 1 μl of the amino acid derivative solution (10 to 50 mg per ml in CH_2Cl_2 or EtOAc) on TLC plates and run in several of the solvents given in Table 3. Assess the purity of Boc amino acids by spraying the developed chromatograms with ninhydrin both before and after deprotection of the Boc group in HCl vapor (see p. 62). The chlorine peptide spray and other sprays for identification of certain groups are also useful for certain derivatives. Approximate Rf's of a number of derivatives in the various solvents are given in

TABLE 3.
Solvents for TLC and paper chromatography.

Solvent[a]	Amino acid derivatives, TLC silica gel	Peptides	
		TLC silica gel	TLC cellulose or paper
A. Chloroform (85), methanol (10), acetic acid (5)	x		
B. Chloroform (95), acetic acid (5)	x		
C. Acetone (98), acetic acid (2)	x		
D. Chloroform (90), methanol (8), acetic acid (2)	x		
E. Chloroform (5), acetone (1)	x		
F. 1-Butanol (15), acetic acid (3), water (12), pyridine (10)	x	x	x P[b]
G. 1-Propanol (84), concentrated ammonium hydroxide (37)	x		
H. Isopropyl ether (6), acetic acid (1), chloroform (3)	x		
I. 1-Butanol (4), acetic acid (1), water (1)		x	x P
J. Phenol (88% liquefied, 3), water (1)		x	x
K. 1-Propanol (2), water (1)			x
L. Pyridine (4), water (1)			x
M. Isoamyl alcohol (7), pyridine (7), water (6)			x P
N. Pyridine (50), acetic acid (30), water (15)		x	x
O. 2-Butanone (10), acetic acid (30), water (25)		x	x
P. Ethyl acetate (5), pyridine (5), acetic acid (1), water (3)		x	
Q. 1-Butanol (65 ml), isopropanol (15 ml), water (20 ml), chloroacetic acid (3 g)		x	x

[a] Parts by volume except where indicated otherwise.
[b] P designates that the solvent is often used in preparative paper chromatography of peptides.

peptides in these solvents (relative to the commonly used solvent I) is as follows (91), for neutral unblocked peptides: N > O > P > I = Q. For very acidic peptides, the relative mobilities are different on paper than on silica gel TLC: for paper, O > N > I = Q ≫ P; for silica gel, N > O > P = I.

Paper electrophoresis (146)

Purity of peptides may be conveniently checked by paper electrophoresis as well as by paper chromatography and amino acid analysis; it is especially useful for peptides containing charged amino acids. Preparative electrophoresis may also be performed, using techniques similar to those described for paper chromatography. A power supply which will furnish 1000 volts at 50 ma is adequate, and can be used for most peptides without cooling. A convenient system uses sheets of Whatman no. 1 filter paper, 4.5 × 22.5 in., sandwiched between pieces of $\frac{1}{4}$ × 5 × 18 in. plate glass. Up to six spots can be applied to such paper strips. Hold the plates together with large office clips (Hunt Clip #4), using four on each side. Before first being used, treat the sides of the glass plates which face the paper to make them water-repellent, by rubbing them with silicone grease; wipe off excess grease. Dip the paper in the buffer, blot well between filter-paper sheets, and spot with the samples, using 1 to 10 μl. Always apply a reference mixture containing picric acid, alanine, aspartic acid, and arginine (1 mg per ml of each) to the sheet. The picric acid provides a visual indication of the progress of the electrophoresis during the run. Rest the ends of the glass plate sandwich on the buffer wells or place a support under the sandwich. Dip the ends of the paper into the buffer wells, making certain that the buffer is at the same level in each well. The time of electrophoresis is 1 or 2 hours at 1000 v, or the time required for picric acid to travel 10 cm from the origin. The *p*H 6.4 buffer has a strong pyridine odor and should be used in the hood. After the current is turned off, remove the sheets from the sandwich and air-dry in a hood. Locate the materials by an appropriate spray. Remove the buffer from the buffer wells, and rinse and drain the wells after each use, especially if they are made of plastic. The buffer may be used several times.

BUFFERS:

1. *p*H 1.9: 88% formic acid (15), acetic acid (10), water (75).

TABLE 5.
Rf's of Boc amino acid p-nitrophenyl esters after TLC on silica Gel G.

Amino acid nitrophenyl ester	Solvent system[a]		
	A	B	C
Boc asparagine	0.63	0.15	0.69
Boc aspartic-β-benzyl ester	0.95	0.60	0.99
Boc glutamic-γ-benzyl ester	0.95	0.58	0.95
Boc glutamine	0.65	0.15	0.75
Boc glycine	0.89	0.55	0.99
Di-Z-histidine	–	0.00	–
Boc phenylalanine	0.95	0.59	1.00
Boc O-benzyl serine	0.95	0.59	1.00

[a]See Table 3 for solvent composition.

TABLE 4.
Rf's of Boc amino acids after TLC in various solvents on Silica Gel G.

Boc amino acid	Solvent[a]							
	A	B	C	D	E	F	G	H
alanine	0.61	0.30	0.75					0.55
nitroarginine	0.30	0.00	0.54					0.06
asparagine	0.21	0.38	0.69					
aspartic β-benzyl ester	0.78	0.29	0.76					
S-benzyl cysteine	0.73	0.42	0.73					
glutamic γ-benzyl ester	0.76	0.22	0.78					
glutamic γ-*p*-nitrobenzyl ester	0.83	0.23						
glutamine	0.36	0.00	0.68					
glycine	0.65	0.21	0.65					0.41
im-benzyl histidine	0.15	0.00	0.09					
im-DNP histidine	0.55							
isoleucine	0.68	0.42	0.73					0.60
leucine	0.70	0.33	0.81					0.64
ε-carbobenzoxy lysine	0.73	0.17	0.68					
α-carbobenzoxy lysine	0.66					0.66	0.66	
methionine	0.69	0.30	0.67					0.54
phenylalanine	0.70	0.39	0.85					0.59
proline	0.63	0.31	0.75					0.51
O-benzyl serine	0.74	0.39	0.70		0.00		0.51	
threonine	0.38	0.04	0.48					0.28
O-benzyl threonine	0.77							
tryptophan	0.50	0.19						0.44
O-benzyl tyrosine	0.77	0.37	0.78	0.50				
valine	0.73	0.39	0.86					0.65

[a]See Table 3 for solvent composition.

Tables 4 and 5. Spotting the derivatives at a low level gives the correct Rf; high levels show the presence of impurities.

TLC OF PEPTIDES. Excellent results have been obtained with cellulose-powder-coated sheets and plates as well as with silica gel plates and sheets. In comparison with paper chromatography, the development times are short (1 or 2 hours) and the small, nondiffused spots give very high resolution and sensitivity. The solvent systems used for paper chromatography are used for cellulose TLC. Some solvents recommended for these and for silica-gel TLC of peptides are given in Table 3. Solvents N, O, P, and Q were recommended by D. Nitecki. Solvent N is for slow-moving compounds; O is the most satisfactory of all the solvents and is very good for fingerprinting. It is about three times faster than 1-butanol-containing solvents. Solvent P is a very fast polar solvent and is suitable only for TLC (not paper). Solvent Q gives about the same mobilities as solvent I, but gives better resolution and is very good for diastereoisomer separations. The migration of

2. *p*H 2.8: 1 *M* acetic acid.
3. *p*H 3.6: pyridine (1), acetic acid (10), water (90).
4. *p*H 5.6: pyridine (2.3), acetic acid (0.6), water (97).
5. *p*H 6.4: pyridine (10), acetic acid (0.4), water (90).
6. *p*H 7–9: tris (hydroxymethyl) aminomethane, 0.05 *M*.

All proportions are in parts by volume.

Identification sprays

The following sprays are useful in identifying amino acids, amino acid derivatives, and peptides. The Sakaguchi spray cannot be used for silica gel TLC plates, but the other sprays can be used for both paper and TLC. (For additional spray reagents, see 96, 123, 126, 146.) Some identifying sprays can be used sequentially, so that a maximum amount of information can be obtained from a single spot (see 24). The sample sizes indicated are those usually spotted for chromatography or electrophoresis. Spots on chromatograms will diffuse and run if the reagent is applied too heavily and the substance spotted is soluble in the reagent. Aqueous reagents should be sprayed very lightly onto chromatograms that bear amino acids or peptides. Dipping is useable only if the chromatographed materials are totally insoluble in the reagent.

Deprotection of Boc amino acids before ninhydrin staining. Dry the TLC sheets or plates after solvent development, and place them in a closed chamber containing a beaker of fresh, concentrated HCl. After 15 min. of exposure to the HCl fumes, heat the plates for 10 min. in a 105° oven. Spray the plates with the buffered ninhydrin reagent while warm; colors usually develop immediately.

Buffered ninhydrin reagent. Spray chromatograms with (or, for paper, dip in) a mixture of acetic acid (3 ml), pyridine (1.5 ml), acetone (96 ml), and ninhydrin (10 mg). After the spray has dried, place the chromatogram in an oven at 105° for 5 min. The ninhydrin reagent should be made fresh each day. The pyridine should be distilled from a flask containing ninhydrin before use. For samples of amino acids, use 1 microliter of 1 mg per ml; for peptides, use increasing amounts in proportion to the chain length.

Unbuffered ninhydrin reagent. A more stable unbuffered reagent, which is adequate for many chromatograms but does not show as many distinguishing colors as the above spray, is a 0.2% solution of ninhydrin in acetone, which is stable for long periods if it is kept refrigerated.

Chlorine peptide spray (*92*). This spray is very useful for protected peptides not visible with ninhydrin, as well as for detecting impurities both in amino acid derivatives and in peptides. A positive reaction is given by almost all compounds which contain N–H groups. All alcohol and acids must be removed from the chromatogram prior to its use. It fails if dioxane has been used for solvent development. Some TLC plates containing a fluorescent indicator have also inhibited the color development.

REAGENTS:

1. Freshly diluted commercial Clorox in H_2O, 1:10 by volume.
2. Ethanol, 95%.
3. 1% KI in H_2O.
4. Freshly prepared *o*-tolidine in 1.5 *M* acetic acid. Mix the *o*-tolidine (about 100 mg to 10 ml of 10% HOAc) and filter through Whatman no. 1 filter paper.

Alternatively, instead of reagents 3 and 4, a solution of 1% soluble starch and 1% KI in water may be used. Bring the water to a boil, and add the soluble starch. Cool the solution to room temperature, and add the KI and dissolve.

PROCEDURE:

Spray thoroughly dried plates or sheets with reagent 1 and let them dry in a hood at room temperature *exactly* 30 min. Spray with reagent 2. After *exactly* 10 min., spray with a 1:1 mixture of reagents 3 and 4 or with the alternate reagent.

The deep blue spots fade rapidly. For samples use 1 microliter of 10 mg per ml.

Conditions must be adjusted carefully if satisfactory results are to be obtained. Excess Clorox, insufficient drying time after the Clorox spray, or insufficient EtOH will cause purple backgrounds. Too long a drying time or too much EtOH will cause faint spots.

A very elegant alternative to reagents 1 and 2 is to spray the chromatogram with a 1% solution of *t*-butyl hypochlorite in cyclohexane (67). The excess of this volatile reagent evaporates readily. This modification has been very useful on silica gel TLC.

Identification of p-nitrophenyl esters. The yellow color of *p*-nitrophenyl esters is intensified by lightly spraying the dried plates with 1 *N* ammonium hydroxide or 0.5 *M* Na_2CO_3.

Sakaguchi spray for arginine (not for silica gel plates).

REAGENTS:

1. 8-hydroxyquinoline or α-naphthol, 0.1% in acetone.
2. NaOBr (0.67 ml Br_2 in 100 ml 1 *N* NaOH).

PROCEDURE:

Dip paper through reagent 1 and air-dry. Lightly spray with reagent 2. Arginine-containing materials are bright orange, fading rapidly. The spots must be outlined immediately with a red pencil or a marking pencil which will write on wet paper. A more sensitive spray, which gives permanent colors, is described by Irrevere (41). Use 1 to 5 microliters of sample containing about 1 mg per ml equivalent of arginine. Color yield may be very low in peptides.

Pauly spray (tyrosine and histidine).

REAGENTS:

1. Sulfanilic acid, 9 g in 90 ml of concentrated HCl.
2. $NaNO_2$, 5% in water.
3. Na_2CO_3, 10% in water.

Store reagents 1 and 2 in the cold (4°).

PROCEDURE:

Mix 10 ml cold reagent 1 and 10 ml cold reagent 2, and let stand at a temperature below 20° for 5 min. Add 20 ml reagent 3 and spray chromatogram lightly. Tyrosine stains pink, histidine orange. Substituents on the imidazole ring of histidine inhibit the color formation. Use 1 to 5 microliters of samples containing about 1 mg per ml equivalent of tyrosine or histidine.

Nitrosonaphthol reagent for tyrosine (123).

REAGENTS:

1. 1-nitroso-2-naphthol, 0.1% in EtOH (acetone *not* recommended).
2. HNO_3, concentrated (70%).

PROCEDURE:

Immediately before use, combine reagent 1 with reagent 2 (9:1) and dip or spray the chromatogram. Let dry 2 or 3 min. at room temperature, then heat 2 or 3 min. at 105°. Tyrosine gives a red color. The spots fade if the heating is prolonged.

Ehrlich spray for tryptophan.

REAGENTS:

1. *p*-dimethylamino benzaldehyde, 10% in concentrated HCl.
2. Acetone.

PROCEDURE:

In a hood, combine before use reagent 1 with reagent 2 (1:4), and spray or dip the chromatogram. Tryptophan-containing materials turn purple after several minutes.

Iodine. Many organic compounds give brown colors when exposed to iodine vapor. Spread iodine crystals on a watch glass in a closed jar and let stand until the jar is filled with vapor. Immerse plates or sheets in the vapor for 5 or 10 min. The brown colors fade rapidly, but can be fixed by spraying with a 1% starch solution after excess I_2 has evaporated.

3

APPARATUS USED IN SOLID PHASE SYNTHESIS

SPECIAL GLASSWARE

Reaction vessels

The reaction vessel originally described by Merrifield (71) is shown in Figure 24. This vessel, in the three sizes indicated, has been widely used and found to be generally satisfactory. When it is used with a rocking device which provides 105° rotation (clockwise from the position shown in the figure), the resin is sufficiently agitated, and if the solvent is added properly (see p. 38), all the resin is satisfactorily washed and brought into reaction. This type of reaction vessel has also been constructed in a very large size (56) suitable for handling up to 50 g of resin, and a microvessel, consisting of a short piece of Teflon tubing shrunk down onto a small fritted disc, with a stopcock at one end and a 10/18 ground joint at the other, has been used (121) for handling a few milligrams of radioactive peptide-resins.

The system of Samuels and Holybee (see pp. 68–70) uses a vessel similar to the one on the Merrifield-Stewart automatic instrument, but with a rocker which provides complete inversion (180°) of the vessel. Enough of the solvents and reagents must always be used to fill the vessel more than half full, so that the walls of the vessel will always be thoroughly washed. This system does not allow coupling reactions to be carried out in very small amounts of solvents in order to accelerate the coupling and minimize side-reactions.

Khosla *et al.* have described a vessel for SPPS which is always kept upright (48). Solvents are added in such a way that the walls are washed down each time, and are removed by a sealed-in filter stick inserted from above and reaching almost to the bottom of the vessel. Mixing is by shaking, and liquids are added through a special funnel that does not require opening the vessel each time. The vessel may be too large (250 or 500 ml) for small batches of resin, and it may be difficult to remove the last traces of solvents by their filtering arrangement.

An upright stationary vessel has been described by Grahl-Nielsen and Tritsch (30). In their system, solvents are metered by measuring pipets and presumably are added in such a way that the entire inner wall of the vessel is washed. The resin is agitated by a motor-driven stirrer, and solvents are removed by suction through a sintered glass disc at the bottom of the vessel.

Sipos and Denning (122) have fitted a tilted (45°) rotary evaporator (Buchler PFE-IBN, continuous feed model) with a synthesis vessel. In operation, the vessel is rotated continually. Solvents and reagents are added through the feed tube at the top and are removed by suction through a magnetic valve below the fritted disc at the bottom of the vessel.

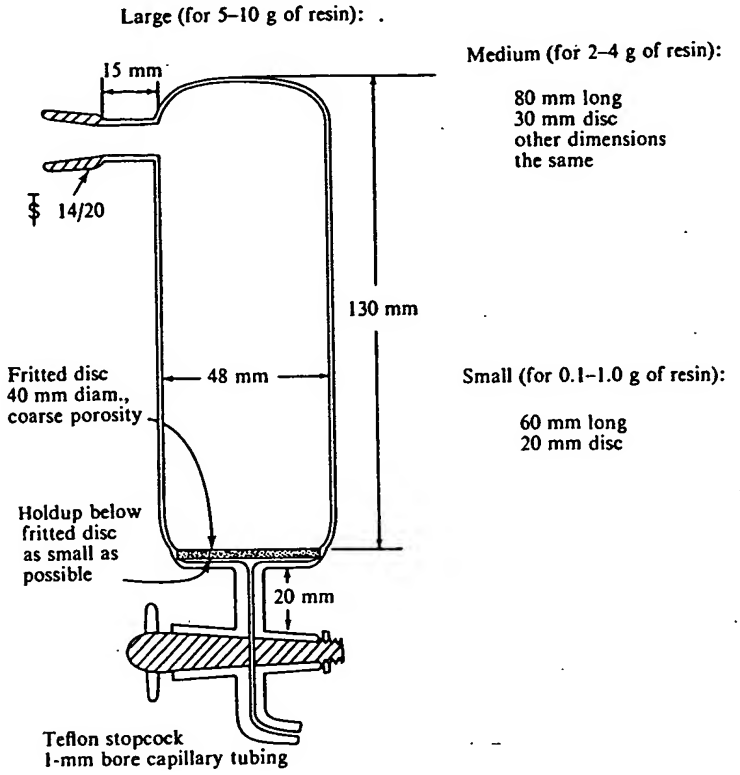

FIGURE 24.
Reaction Vessels.

Many different satisfactory systems of glassware for SPPS could doubtless be devised, and each might possess certain advantages. We consider that an ideal vessel or system would: (1) allow for reasonable variation in batch size —at present different sizes of vessels are necessary; (2) provide for rapid and thorough suspension of the resin, so that all beads are in good contact with the solvent without being subjected to any grinding action, such as is caused by magnetic stirrers; (3) provide for rapid and convenient addition of solvents in such a way that vessel walls are washed down, unless this is provided for by the agitation system; (4) allow different solvent volumes to be used at will with equal efficiency of resin contact; (5) provide for convenient removal of resin samples during the synthesis; and (6) provide for rapid and complete solvent removal.

Cleavage vessels

Although the peptide can be cleaved from the resin with HBr-TFA in the synthesis vessel, it is generally preferable to remove the peptide-resin from the synthesis vessel and dry it before cleavage, since doing so allows the amount of peptide incorporated to be estimated from the weight gain, and also conveniently allows part of the peptide-resin to be stored for future use. The peptide can be conveniently cleaved from the resin with HBr-TFA in one of the vessels